Microbial quality control management in industry:
Approaches for assessing bioburden and community composition

By Adrienne Davies

Thesis submitted for the degree of Professional Doctorate

School of Life Sciences, University of Warwick

Sponsored by

Omya Microbiology, Omya International AG

October 2015

Sections and figures containing sensitive information have been obscured or removed.

Contents

Acknowledgements

I owe thanks to a large number of people at Warwick for their support over the years. Firstly, thank you to my supervisors Dr. Crawford Dow and Dr. Kevin Purdy for their guidance and wisdom throughout my PhD. Thank you Crawford for your persistent optimism, encouragement, and understanding. I am grateful for the opportunities provided to me during this project, all of which would not have existed without your assistance. Thank you to my wonderful friends and colleagues who helped me keep my sanity, made me laugh, and shared my passion of baking and eating cakes: the Purdy lab, the Scanlan and Bending groups, and the office of C-126 (especially Piv and Kathryn). Thanks also to my dear friends Krisztina and Mark for their unrelenting cheerfulness and thought-provoking discussions. Lastly, thank you to my colleagues on the MSc. BBBM course for their support: Dr. Charlotte Moonan, Dr. Steve Hicks, Dr. Robert Old and many others.

I would like to thank Omya International AG for the financial support and use of their laboratories and equipment in Oftringen for much of the research. I am grateful to my colleagues in Omya Microbiology over the years for making me feel welcome in the lab: Amanda, Daniela, Helen, Karin, Katharina, Klaus, Koni, Martina, Regula, and Vera. I would also like to thank Lisbeth Siegrist for her kindness and hospitality during my stays in her home. Thank you to my industrial supervisors past and present for their insight and enlightening discussions: Dr. Patrick Schwarzentruber, Dr. Nicola Di Maiuta, Dr. Simon Urwyler, and Dr. Joachim Glaubitz. Thank you also to the laboratory managers at the European plants for their cooperation in completing the survey: J. Dyrhaug, L. Pappalardo, and T.G. Sollund. I am especially indebted to and appreciative for the assistance and commitment of Fred Voorbraak and Ed Brugman at the Omya Moerdijk plant in the Netherlands.

Finally, I would not have made it through this time without the love and support of my family and friends in Toronto. To my parents, George and Suk, thank you for your belief in me and for challenging and inspiring me to achieve more than I ever thought possible.

Declaration

This thesis has been submitted to the University of Warwick in support of my application for the degree of Professional Doctorate. I hereby declare that the work presented here is my own and has not been previously submitted to this or any other institution for any degree, diploma or other qualification.

Adrienne Davies
October 2015

Summary

Microbial contamination of industrial products and processes can endanger the health and safety of consumers and workers and negatively affect businesses by causing product spoilage and decreases in process efficiency. Major microbial contamination events can result in financial losses from product recalls, unplanned production downtime, loss of sales and litigation. Effective microbial monitoring is essential for detecting the presence of microorganisms and helps businesses control and maintain product quality. This thesis first examines the challenges in achieving accurate assessments of microbial load, which is defined as the quantity of viable microorganisms within a given sample matrix (Chapter 2). Classical culture techniques remain the most widely utilized and approved methods but their major shortcomings have limited their applicability in Quality Management systems employed to control contamination throughout product processing, handling, and distribution. Issues such as the long incubation times to achieve a result, the selective bias of culture conditions on species recovery, and the inability to detect damaged or injured cells are discussed. Rapid microbiological methods (RMMs) offer increased sensitivity and accuracy, can deliver results in near real-time, and could enable more effective and proactive management of microbial contamination problems. Nevertheless, their uptake in industry has been hindered by their higher operating costs and time-consuming proof-of-equivalency studies to the reference culture methods, which are required for approval by the relevant regulatory bodies. An overview of commercially available RMMs is provided and the suitability of each method is application- and industry-dependent.

An industrial case study of microbial monitoring methods was performed with Omya AG (Switzerland) the top global producer of calcium carbonate (CC) slurries (Chapter 3). CC slurries are used as manufacturing fillers in a wide range of industries including paper, paints, construction and food. High microbial loads have been shown to adversely effect slurry quality and performance and negatively impact on customers' finished products (Schwarzentruber, 2003). Quality Assurance (QA) managers rely on culture dip slides for detecting microbial load and directing biocide treatment to manage excessive population growth. RMMs offer Omya the potential for cost savings from effective biocide management and could help QA managers uncover and remedy microbial-related product spoilage sooner. The viability-based RMM CellFacts II (CFII) (CellFacts2014 Ltd., UK) combines electrical flow impedance and viable cell specific fluorescent staining to produce an accurate, real-time measurement of the contaminating population. To improve its

usability and desirability to Omya the technology was optimized by streamlining sample preparation steps, the cost per test was reduced, and data presentation and interpretation was simplified. During this study CFII showed increased sensitivity and reliability compared to dip slides and highlighted the differences in preservation efficiency and bioburden levels in slurries preserved with biocide blends or by pH-stabilization.

Although pH stabilizers are added to slurries during production, Omya still relies upon biocides for managing any uncontrolled microbial growth detected during product storage or upon delivery to customers. The biocide-preserved slurries that were analyzed were maintained at pH 8.5-9.0 while the pH-stabilized slurry were pH 9.5-10.0. It was hypothesized that this 1-log difference in pH impacted upon microbial community composition and it was necessary to ensure that these populations were still susceptible to post-production biocide treatments. A multifaceted approach was used to characterize the communities and assess the coverage and limitations of each method (Chapter 4). Samples were collected and microbial load was determined by plate culture and CFII, and population diversity was elucidated by the recovery of species in culture and by 16S rRNA gene analysis by Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and sequencing on the Illumina MiSeq (USA). A comparison of direct and indirect DNA extraction methods was performed to observe the efficiency and differences in DNA recovery from microbial species. In addition, DNA from the viable population was selectively amplified by treating isolated microbial cells with propidium monoazide (PMA), a dye that enters non-viable cells, binds DNA and inhibits PCR amplification. Biocide-preserved slurry showed lower diversity in culture compared to pH-stabilized slurry but displayed a higher number of operational taxonomic units (OTUs) by T-RFLP and sequence analysis in the microbiome analysis platform Quantitative Insights in Microbial Ecology (QIIME). The microbial communities were significantly different with *Pseudomonas* spp. associated with biocide-preserved slurry and alkaliphilic *Bacillus* spp. dominating in pH-stabilized products. The culture media conditions were inadequate for recovering the highly abundant alkaliphilic population in pH-stabilized slurry and future culture work must be optimized on alkaliphilic media. 16S rRNA gene analyses produced a more truthful representation of the community and DNA extracted from PMA-treated cells revealed changes in population structure after 48 hours of incubation and in response to biocide addition. Slurry populations are dynamic; regular sample collection at various time points is necessary to achieve accurate detection and monitoring of microbial communities.

List of Figures

List of Tables

List of abbreviations

AK	OmyAK biocide
AMP	2-amino-2-methyl-1-propanol; Corrguard-75
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
ATGU	Austria Gummern plant
ATP	Adenosine triphosphate
BNPD	Bronopol; 2-Bromo-2-nitro-propan-1,3-diol
bp	Base pairs
BPR	Biocide Products Regulation
CC	Calcium carbonate
CF	CellFacts 2014 Ltd.
CFII	CellFacts II
CFR	Code of Federal Regulations
CFU	Colony forming units
CHA	Chlorhexidine
CMIT	5-chloro-2-methyl-4-isothiazolin-3-one
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
$diSC_3(5)$	3,3-dipropylthiadicarbocyanine iodide
EC	European Commission
ECHA	European Chemicals Agency
EDDM	Ethylenedioxy dimethanol
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
ELFA	Enzyme-linked fluorescence assay
ELISA	Enzyme-linked immunosorbant assay
EMA	Ethidium monoazide
EPA	Environmental Protection Agency
FA	Facultative alkaliphile
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
FIFRA	Federal Insecticide Fungicide Rodenticide Act
FISH	Fluorescence *in situ* hybridization
FSIS	US Food Safety and Inspection Service
FSMA	Food Safety Modernization Act
GCC	Ground calcium carbonate
gDNA	Genomic DNA
GMP	Good manufacturing practice
GTA	Glutaraldehyde
HACCP	Hazards Analysis and Critical Control Points
ISO	International Standards Organization
IST	Isothiazolones
ITAV	Italy Avenza plant
LAMP	Loop-mediated isothermal amplification
LPS	Lipopolysaccharide
MALDI	Matrix Assisted Laser Desorption Ionization-Time of Flight
MIC	Minimum inhibitory concentration
MIT	2-methyl-4-isothiazolin-3-one
M-L	Muramic-δ-lactam
mPCR	Multiplexed PCR
MRL	Maximum residue limit
NASBA	Nucleic acid sequence-based amplification
NLMJ	Netherlands Moerdijk plant
NOME	Norway Molde plant
NP	Neutralophile
OA	Obligate alkaliphile
OMP	Outer membrane protein
OPP	ortho-phenylphenol
PCA	Plate count agar
PCC	Precipitated calcium carbonate
PCR	Polymerase chain reaction
PG	Peptidoglycan
PMA	Propidium monoazide
QA	Quality Assurance
QAC	Quaternary ammonium cations
QIIME	Quantitative Insights in Microbial Ecology
RFU	Relative fluorescence units

RLU	Relative light units
RMM	Rapid microbiological method
Su13	Summer 2013
Sp14	Spring 2014
SPR	Surface plasmon resonance
T-RFLP	Terminal restriction fragment length polymorphism
TSA	Tryptic soy agar
TVC	Total viable count
USD	US dollars
VBNC	Viable but not culturable
WHO	World Health Organization

Chapter 1

Introduction

Introduction

Microbial load, or bioburden, is defined as the quantity of viable microorganisms within a given sample matrix. Effective bioburden monitoring in industry is necessary to prevent the negative consequences of microbial contamination. Traditional methods for enumerating bacteria are imprecise and plagued by long delays for achieving a result. Industry requires tools with increased accuracy and sensitivity, and rapid microbiological methods (RMMs) may provide an improved solution. When microbial load is no longer efficiently controlled by a given antimicrobial strategy, in-depth characterization of the community can provide insight into population dynamics.

1.1 Aims of the thesis

The aims of this project were to:

- Examine the impact of microbial contamination of a product on a business and outline the strategies for controlling contamination during production and by use of biocides for inhibiting microbial growth
- Provide an overview of technologies for rapid microbiological testing and discuss the implementation barriers in industry
- Use Omya AG as a case study to examine the challenges in controlling and preventing microbial spoilage of an industrial product such as calcium carbonate slurries
- Evaluate the effectiveness of different chemical product preservation strategies
- Optimize and transfer a rapid microbiological method to an industrial scale and evaluate the cost savings opportunities in quality management
- Characterize microbial community composition differences in pH-stabilized and biocide-preserved slurries by using classical culture methods and 16S rRNA gene analysis by T-RFLP and amplicon sequencing

Chapter 2

Microbial quality control management in industry

2.1 Introduction

Microbial contamination is a major concern in many industries. Excessive microbial growth can cause the deterioration of equipment and machinery, decrease process efficiency in manufacturing, negatively impact upon end-product quality, and most importantly, endanger the health and safety of consumers and workers. The food, healthcare, pharmaceutical, and personal care products industries are the most at risk for causing severe illness or death since their products are ingested or applied topically. Business operators attempt to identify the presence of pathogens or high bioburden levels before the goods reach customers, but in some instances the issue only becomes apparent after illnesses are reported (Karanam *et al.*, 2008; Sutton and Jimenez, 2012). This is a costly consequence of poor microbial monitoring and ineffective Quality Assurance (QA) management systems. To decrease the risk of contamination, international administrations of the Food and Agricultural Organization (FAO), World Health Organization (WHO) and International Standards Organization (ISO) have provided guidelines for good manufacturing practice (GMP) and recommended operating procedures for controlling and minimizing contamination (Table 2.1). Regional regulatory bodies such as the US Food and Drug Administration (FDA) and the EU's European Food Safety Authority (EFSA) have set more specific regulations and restrictions for products during processing, manufacturing, and packaging (Table 2.1). Effective microbial quality management includes aspects of plant hygiene maintenance, regular and accurate monitoring of microbial load in raw materials and finished products, and control strategies for correcting problems when they occur (Hiom, 2004). However, even with oversight and a regulatory framework in place microbial contamination continues to pose a hazard to consumers and negatively impact upon businesses. Most failures are the result of inadequate process controls and methods for preventing and detecting microbial growth and a lack of GMP (Karanam *et al.*, 2008). Most of the approved techniques in industry for measuring bioburden levels and identifying pathogens are based on cultivation methods (Shama and Malik, 2013; Hiom *et al.*, 2013; Sutton, 2011). The main limitations of culture-based approaches are low sensitivity and the long length of time required to achieve a result (Peris-Vicente *et al.*, 2015; Jasson *et al.*, 2010; Bottari *et al.*, 2015). Aspects of the culture environment including nutrient-content, temperature, and atmosphere place constraints on the microbial population. Only organisms capable of growing under the defined conditions and those in an active physiological state will recover on media. This is important to consider when evaluating the effectiveness of preservation treatments. Microbial growth can be inhibited by exposure to physical stresses such as changes in temperature or pH or

by chemical stress from exposure to antimicrobial compounds. Dormant and injured cells are slow to recover on media (if at all); therefore, culture-based methods cannot provide an accurate measurement of viable cell counts (Sutton, 2011; Karanam *et al.*, 2008; Simões *et al.*, 2005; Zhao *et al.*, 2014). Novel technologies can provide near real-time detection of microbial load with increased sensitivity. Rapid microbiological methods (RMMs) could help businesses improve product quality, reduce overall quality management costs, and lessen or eliminate the release of contaminated products to the market (Jasson *et al.*, 2010). Productivity could also be increased from faster detection and correction of uncontrolled growth or unwanted microorganisms (Velusamy *et al.*, 2010).

Table 2.1 - General International, European and American guidelines and regulations for food product manufacturing and microbial limits

International	Overview
FAO/WHO Codex Alimentarius Commission CAC/RCP 1- 1969, rev. 4-2003	Principles of Hazards Analysis and Critical Control Points (HACCP) and guidance for its application
ISO 22000:2005	List of international standards for food safety management
Europe	
REGULATION (EC) No 852/2004 on the hygiene of foodstuffs	General and specific hygiene rules for business operators involved in preparation, handling, storage and packaging along the food chain; implementation of procedures based on HACCP
REGULATION (EC) No 2073/2005 on microbiological criteria for foodstuffs	Microbial limits for microorganisms and their metabolites and general food safety and process handling criteria of foodstuffs
US	
Code of Federal Regulations Title 21 (21 CFR) Part 120 - HACCP systems	General provisions for the manufacture of foods, pathogen reduction processes and verification
21 CFR Part 110 - Current good manufacturing practice in manufacturing, packing, or holding human food	General provisions for facilities, equipment, process controls and defect action levels

2.1.1 Aims and objectives

The aims of the following discussion were to:

- Examine the impact microbial contamination can have on a business by observing recent high profile pathogen outbreaks
- Outline the strategies for controlling contamination of products, including the implementation of a Hazards Analysis and Critical Control Points (HACCP) plan and the use of biocides for inhibiting microbial growth
- Review the mechanisms of biocide resistance in microorganisms and the inadequacies of culture methods in assessing antimicrobial effectiveness

- Provide an overview of technologies for rapid microbiological testing and discuss the implementation barriers in industry

2.2 Consequences of microbial contamination in business

When goods are suspected and/or confirmed to be contaminated a company will recall the products to prevent further risks or incidences of illness. An overview of significant microbial contamination and recall events is reported in Table 2.2. These all involved the detection of pathogenic organism such as *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli*, and *Clostridium botulinum* in foods and food products. Personal care products are considered to be non-sterile but are also subject to bioburden limits and must not contain certain "objectionable organisms". Some restrictions are dependent on the intended use of the product (e.g. wipes, nasal spray, mouthwash) and the intended recipients (adults, infants, the infirm). In a review of product recalls in the US from 2004-2011 it was revealed that 34% of recalled non-sterile products contained *Burkholderia cepacia*, 19% had fungal contamination, and 9% contained *Bacillus cereus* (Sutton and Jimenez, 2012).

In a survey of major grocery manufacturers in the US, 81% of respondents described the financial impact of recalls as significant to catastrophic and highlighted the costs from business interruption and product disposal to be the highest (Grocery Manufacturers Association, 2011). If the infected product stock affects a large volume it could take time to trace, destroy, and replace it on the market. When the source of contamination is unknown, production and manufacturing is stopped while inspections are carried out. Government agencies are often required to approve a return to production and additional downtime for decontamination brings further delays. Some companies do not survive the expenses of recall logistics and interruptions to operations, while others fall from the loss of sales, customer reimbursement and resulting liabilities. In the Jensen Farms and Peanut Corporation of America examples in Table 2.2, the companies declared bankruptcy after product recalls and investigations by the FDA. In both instances the business owners were fined and faced lengthy prison sentences for selling contaminated products. The Jensen Farms' case was the result of incompetence, poor agricultural and management practices, and ineffective control and cleaning measures (US FDA, 2012). The FDA's findings of the Peanut Corporation of America investigation were more damning. In addition to poor plant hygiene and maintenance, three business operators were found guilty of knowingly selling

contaminated peanut products to suppliers and fabricating certificates of analysis and laboratory results for the products (US Department of Justice, 2014).

Large multinational companies tend to be more resilient in the face of recalls owing to strong brand names, sizeable financial resources, good regulatory compliance and effective crisis management. These companies have invested time and money into building up their image and product brands, their reputations, and in garnering customer trust and loyalty. They understand the value in protecting their brand, the importance of transparency, and the need for swift action when product contamination is suspected. Companies such as the Coca-Cola Company, Johnson and Johnson, Bridgestone Corporation and Toyota have survived large-scale product recalls over chemical contaminants or defective parts. Manning (2007) highlighted Cadbury's rapid response in implementing increased product testing and changing plant configurations and cleaning regimes as the basis for retaining public confidence during the *Salmonella* contamination announcement (Table 2.2).

The extent of damage due to a recall is also dependent on the magnitude of the problem, namely the amount of product affected and the consequences to consumer health. New Zealand dairy manufacturer Fonterra suffered major losses from recalls instituted after fears over *C. botulinum* contamination in concentrated whey protein. Some Asian countries imposed import bans and Danone pulled 70,000 cans of whey-containing infant formula off shelves. Further testing revealed the whey was negative for *C. botulinum* but positive for *C. sporogenes*, an organism that can lead to increased product spoilage but does not produce neurotoxins. The losses would have been more extensive with a positive result for botulinum-producing *Clostridia* and if adverse health effects had been reported. Furthermore, Danone is seeking third-party liability for the loss of revenue and loss of future revenue due to damage to its brand image and reputation. While a "brand" is often described as being an intangible business asset, some European organizations have included the change in value of their brand in profit and loss statements (Manning, 2007). Consumers associate brands with a particular level of quality, confidence, reliability, and safety. After a few consecutive years of revenue growth Danone posted a loss in the year following the recall. They attributed this to the scandal and are pursuing legal action for compensation (Table 2.2).

Table 2.2 - Microbial contamination prompting product recalls

Product	Company	Year	Country	Recall and consumer health risk	Financial impact (USD)
Cadbury chocolate	(Then) Cadbury Schwepps	2006	UK	*Salmonella* contamination in > 1 million chocolate bars. 180 illnesses reported (Carroll, 2006)	$50 million
Tomatoes/peppers	Various	2008	USA	*Salmonella enterica* Saintpaul outbreak falsely linked to tomatoes then traced to Mexican-grown peppers	Tomato industry lost > $100 million
Peanuts	Peanut Corporation of America	2008	USA	Peanuts contaminated with *Salmonella typhimurium*. Affected >200 companies and >2000 products. 700 illnesses reported, may have contributed to 9 deaths (Hussain and Dawson, 2013)	Industry loss > $1 billion. Peanut Corporation of America declared bankruptcy. The CEO is still awaiting sentencing (due in September 2015) and could be facing life in prison (Snyder, 2015)
Eggs	Wright County Egg Farm	2010	USA	500 million shell eggs recalled due to *Salmonella enteriditis*. 2,500 illnesses reported	Unspecified losses but a price drop for eggs cost the industry $100 million in the month of September alone (in 2010)
Cantaloupe	Jensen Farms	2011	USA	*L. monocytogenes* contamination. 33 deaths and hundreds of illnesses	Company declared bankruptcy. Owners faced prison time but were fined and sentenced to 5 years probation in 2014 (Sanchez, 2014)
Cucumbers/sprouts	European farmers	2011	EU member states	*E. coli* contamination falsely linked to cucumbers then traced to fenugreek seeds imported from Egypt. 50 deaths and > 4,000 illnesses	Loss estimated at $1.3 billion with an additional $236 million in emergency aid payments (Knüsli *et al.*, 2015)
Whey protein concentrate	Fonterra	2013	New Zealand	Inaccurate report of the presence of *C. botulinum.* Recall affected products and manufacturers worldwide	Loss > $100 million. Danone Nutricia seeking damages of up to €350 million (BusinessDesk, 2015)

Table modified from Steves (2014)

Fonterra was criticized over the confusion and anxiety caused by the contamination results. Product testing was outsourced to a contract laboratory and Fonterra reportedly took three months to issue the precautionary recall. Independent testing at local and international laboratories ordered by the New Zealand government disproved the results within one month (Gray, 2013). Critics questioned the effectiveness of the microbial quality monitoring process. On the one hand, the delay in acting on the supposed *C. botulinum* results was unacceptable since manufacturing continued for a period and product was released to the market. On the other hand, the initial test results were false. The recall not only reflected poorly on Fonterra but on New Zealand itself. The opposition party's shadow minister for the Ministry of Primary Industries at the time stated " ...our failure to ensure the highest standards of testing, monitoring and auditing means the damage has been done to New Zealand's international reputation" (Gray, 2013). In order to ensure that circumstances and mistakes are not repeated, governments and regulators must continually re-evaluate and modify legislations to better protect their citizens.

The US FDA had been aspiring to have more authority over facility shutdowns, in issuing recalls, and in detaining foods believed to be contaminated. Their role had been perceived as being reactive to problems instead of proactive in preventing contamination, enforcing regulations and protecting public health. In 2011 US Congress enacted the Food Safety Modernization Act (FSMA) that enabled the FDA to:

1. Issue a mandatory recall when companies fail to voluntarily recall unsafe food and food products. (Previously a court order was required);
2. Enforce the utilization of hazards analysis and critical control points at facilities (HACCP) (discussed in the next subsection);
3. Set quality performance standards with respect to monitoring quality and test outcomes;
4. Engage in more frequent site inspections for facilities deemed high-risk for contaminant problems;
5. Order the shutdown of facilities for violating safety and quality standards;
6. Design a traceability program to identify sources of contamination in processed and prepared foods;
7. Require certification for and refuse admission of imported foods to the US;

An outbreak of *Salmonella enterica* serovar Bredeney across 20 states that left 42 people ill in September 2012 was traced to peanut butter manufactured by Sunland Inc.. The FDA shutdown the facility in November 2012 after investigations revealed improper storage and handling of raw materials and equipment, inadequate food

testing and screening methods, and no documented evidence of cleaning regimes (US FDA, 2013). The company was also barred from distributing food products into the marketplace. It was the first instance of the FDA directing its authority under the FSMA. The facility remained closed until early-2013 when the recommended amendments were made to the plant layout, new equipment was purchased, an independent sanitation expert was hired, and proper sanitation controls and environmental monitoring procedures were in place. Despite resuming operations the company filed for bankruptcy shortly after. Sunland Inc. posed as an example to others of the consequences of non-compliance. Action is taken swiftly and the punishment is severe when regulations are not met. The strategy for increasing profits often comes at the expense of decreasing costs during manufacturing and processing. However, end-product failures can cost the company more than investments in upstream quality monitoring and preventative measures.

Total quality costs can be categorized into costs for prevention, appraisal, internal failure and external failure (Table 2.3) (Omachonu *et al.*, 2004). As the name suggests, activities under the prevention component include those that prevent problems from occurring or developing during product processing. Appraisal takes place throughout production and ensures product conformance to specifications. Internal failures are problems detected before delivery to the customer while external ones are detected after. The situations resulting in recalls discussed above are the result of non-conforming products and external failures. In some cases the businesses did not recover after sustaining significant failure costs. Many authors have discussed the concept of "quality costs" and the balance between the different classifications including Chauvel and Andre (1985), Harrington (1987) and Carr and Ponoemon (1994) in Omachonu *et al.* (2004). Investment in prevention activities was found to positively influence profit margins and the combination of increased prevention and appraisal costs could decrease total quality costs (Chauvel and Andre, 1985). However, investment in appraisal alone did not reduce the total number of product failures, which leaves a company's reputation at risk (Harrington, 1987; Chauvel and Andre, 1985). In a study of quality costs in paper manufacturing, costs towards prevention were found to be the least expensive cost component and a retroactive analysis showed that the combination of internal and external failure costs always exceeded that of prevention and appraisal (Carr and Ponoemon, 1994). Therefore, in order to minimize or eliminate total failure costs companies must invest in prevention strategies including good plant design, maintaining good plant hygiene and use of preservatives, and execute more appraisal activities such as effective environment and product testing regimes.

Table 2.3 - Categories encompassing total quality costs

Prevention	Appraisal	Internal failure	External failure
Costs towards preventing product failure: • Designing HACCP plan • Preventative maintenance • Application of good plant hygiene • Product preservation or disinfection	Costs incurred to ensure product quality conforms to specifications: • Raw materials inspection • In-process and end-product testing (including consumables and equipment used in monitoring)	Costs sustained to correct problems before shipment to the customer: • Reworking or treatment to recover product quality • Disposal costs for unrecoverable and defective products	Costs incurred when problems are detected after shipment to the customer: • Returned material and logistics • Complaint adjustment costs • Liabilities and litigation • Lost sales

Adapted from Kim & Nakhai (2008)

2.3 Strategies for managing and controlling contamination

2.3.1 Implementing process controls and effective monitoring

Hazards Analysis and Critical Control Points (HACCP) is an internationally applied quality management system that functions to minimize, control, and eliminate hazards during the production, manufacturing, handling, and storage of foods. The seven principles of HACCP are listed in Figure 2.1. The first stage involves a critical analysis of the process in question and the identification of potential risks and hazards to the procedure or final product. In this instance the hazards are characterized as microorganisms that have the potential to enter the product and cause injury or illness if they are not adequately controlled. The second principle involves the identification of critical control points (CCPs) within the process to prevent, reduce or eliminate bioburden. Some examples of CCPs are heat sterilization or surface disinfection steps, or the addition of preservatives. In the third stage the critical limits of a CCP are identified. The limits are typically a visible or quantifiable measurement such as temperature or pH to facilitate in-process monitoring (the fourth principle) and the application of corrective actions when there is a deviation from critical limits (the fifth principle). The success of controlling, monitoring, and correcting CCPs is verified by additional testing (principle six) and by reviewing well-documented and stored records (principle seven). When local authorities investigate or audit a facility and check for HACCP compliance they review the documentation. From the perspective of the regulators, if the work and the results are not recorded then they did not take place. The implementation of HACCP allows businesses to demonstrate their understanding of and ability to effectively detect and control CCPs and hazards when they arise in their processes.

In the industrial examples listed in Table 2.2, the companies either possessed no or a poor HACCP plan. They all failed to prevent contamination, some failed to detect it, and all failed to apply corrective measures.

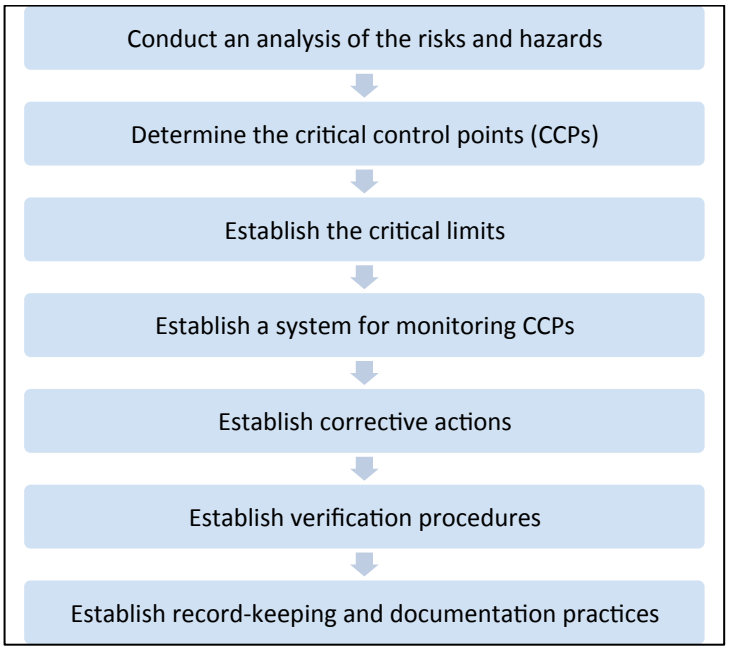


Figure 2.1 - The seven principles of HACCP.
Modified from US FDA (1997)

Another important aspect included within HACCP is plant hygiene and equipment maintenance. The conveyor belts in the Jensen Farms cantaloupe facility were not cleaned and tested positive for *Listeria* (US FDA, 2012) and investigations at Sunland Inc. revealed 28 *Salmonella*-positive samples swabbed from the plant environment (US FDA, 2013). Plant design and plant hygiene plays a crucial role in minimizing and controlling contamination. Having an effective housekeeping schedule to keep surfaces clear of microbial build-up will reduce the risk of contamination during storage and production. The design of equipment and materials and the layout of the facility can also contribute to contamination problems if surfaces are difficult to clean or provide an environment that encourages microbial growth (such as sharp bends in piping or dead spots in large reactors). Additional considerations for plant design, sanitation, and raw materials handling is provided in Table 2.4. Some industrial products have a CCP where preservatives or biocides are added during production. Maintaining good hygiene practice mitigates the risks of in-process contamination and could improve the efficiency of preservatives in controlling microbial quality in the end product.

Table 2.4 - Control measures for plant design, plant hygiene and product hygiene

Plant design	
• Avoid sharp angles and dead spots in piping and large mixing tanks where product can build-up and cleaning is difficult • Avoid long, straight stretches of piping that impedes proper drainage and emptying • Avoid porous surfaces such as concrete in tanks • Although plastic surfaces present minimal risk of corrosion they have a higher risk for biofilm formation than stainless steel • Use closed containers for storage and/or processing where possible • Facility layout should minimize cross-contamination of products and processes by separating "clean" and "dirty" areas	
Plant hygiene	**Product hygiene**
• Develop a regular housekeeping and sanitation schedule and keep clear records • Keep the work environment and surfaces clean (floors, walls, ceilings, pipelines) • Check the compatibility between disinfectants and cleaning agents with the materials being cleaned • Regularly clean splash zone areas such as the headspace above the bulk phase of product within a reactor • Inspect and clean and/or replace membranes in filters regularly • Surfaces should be cleaned with a disinfectant or biocide rinse • Surface swabs should be regularly monitored for microbial growth to check the effectiveness of cleaning regimes • Personnel should be well trained in hygienic practices and should follow the requirements for personal hygiene	• Materials should be stored in covered or closed containers and protected from air, moisture, environmental contaminants and temperature extremes • A "first-in, first-out" approach will ensure shorter materials storage times and reduce the risk of excessive microbial growth • Recycled water should be treated before being reused in the system • Regular mixing or agitation of wet products in reactors will ensure preservatives remain evenly distributed throughout the product and reduce the risk of anaerobic growth • Regular microbial monitoring of raw materials will inform on the bioburden level before entering production

Adapted from Schwarzentruber (2007)

2.3.2 Disinfection and preservation by use of biocides

Microbial growth can be inhibited in industrial products by physical and chemical methods or a combination of the two. The effects can be microstatic where microbial growth is inhibited but the cells are not killed, or they can be microbicidal where the cells are inactivated and killed. Some examples of microstatic and microbicidal treatments are listed in Table 2.5. The type of preservation method applied is dependent on the product, its physical characteristics and the desired shelf-life, the regulations in that business sector, and economics. For instance, citrus fruits, milk, body lotion, printing paper, wall paint, and wastewater undergo different physical and chemical treatment methods to prevent microbial growth. Unlike physical methods spraying or dipping fruits and vegetables in biocide solution has minimal effect on their physical and organoleptic properties. Techniques such as pasteurization are suitable for preserving beverages but not financially feasible

nor sufficient to provide long-lasting protection in cosmetics or personal care products requiring a long shelf-life. This discussion will focus on the use of antimicrobials and biocides for preserving industrial products.

Table 2.5 - Techniques for controlling microbial growth

Objective	Method of preservation	Examples
To reduce or inhibit growth (biostatic)	Low temperature	Freezing or refrigeration during processing or storage
	Desiccation	Lyophilization/freeze-drying; high-heat evaporation
	Oxygen limitation	Vacuum packing
	Nutrient restriction	Water-in-oil emulsions
	Osmotic pressure	High/low pH; high-salt; high-sugar
	Chemical preservation with antimicrobials	Phenolics; alcohols; biguanides; halogens; surfactants; heavy metals; aldehydes; peroxygens
To inactivate cells (biocidal)	Heating	Sterilization by boiling, steam, or dry heat; pasteurization; ultra high temperature
	Radiation	Gamma rays; x-rays; ultraviolet
	Pressurizing	High hydrostatic pressure
	Electroporation	High voltage electric discharge
	Manothermosonication	Ultrasonication combined with high heat and pressure
	Biocides	Applying higher/lethal concentrations of antimicrobials

Adapted from Gould (1996)

2.3.2.1 Biocide market overview and regulations of biocidal products

The global biocide market was valued at over $7.3 billion USD in 2012 with North America and Asia accounting for 41% and 30% of the total revenues respectively (APCJ, 2014). Alongside strong economic growth in Asia and Latin America the market is expected to reach $10.7 billion by 2020 (European Coatings, 2014). The largest uses for biocides are in water treatment, wood preservation, and the manufacturing of pulp and paper, paints and coatings, food and beverages, and personal care products. Biocidal compounds must be registered with regional regulatory agencies and are subject to different restrictions within each business sector. The regulatory systems and their procedures are complex and beyond the scope of this study. Briefly, in the US biocides must be registered with the Environmental Protection Agency (EPA) under the Federal Insecticide Fungicide Rodenticide Act (FIFRA) and in the EU they are registered under the Biocidal Products Regulation (BPR, Regulation EU 528/2012) with the European Chemicals Agency (ECHA). The registration process requires complete safety and hazard assessments of the active substances before they are authorized for sales and distribution in the marketplace. For some industrial applications this general

information may be sufficient for receiving approval for an intended use. Other applications may need additional tests to demonstrate compatibility, safety, and efficacy; as a result, biocides can be approved in some applications but not in others, or they may possess different allowable concentration limits in the final products.

An example of this is the registered pesticide ortho-phenylphenol (OPP). OPP has the regulatory approval for use as an industrial surface disinfectant, a post-harvest fungicide for citrus fruits and pears, for the protection of mineral dispersions, paints, and metal working fluids, and as a preservative for textiles, leather goods and wood products. The acceptable daily intake of OPP is 0.4 mg/kg of bodyweight per day according to the WHO/FAO (Codex Alimentarius, 2013). As a disinfectant for surfaces in food manufacturing the concentration of the solution was not to exceed 400 mg/kg (400 ppm), but recent toxicity testing adjusted the limit up to 4200 mg/kg (US EPA, 2006). In the surface treatment of citrus crops and pears the EU authorizes a maximum residue limit (MRL) of 5 mg/kg fruit but in other fruits, vegetables, or nuts the MRL is only 0.05-0.10 mg/kg (Regulation EC No. 737/2014). The WHO/FAO and EPA are less restrictive recommending MRLs of 10 and 25 mg/kg fruit in citrus fruits and pears respectively (Codex Alimentarius, 2013; US EPA, 2006). Although it is approved for use as a chemical additive in paper packaging materials for food, the acceptable MRL in the finished product is even lower. This topic is explored in more detail in the next Chapter.

2.3.2.2 Biocidal targets in bacteria and mechanisms of action

Biocides can be classified into two groups based on their mechanism of action: membrane-active agents and electrophilically active compounds (Figure 2.2). Depending on the concentrations used, biocides can exert bacteriostatic or bactericidal effects on a microbial population (Figure 2.3). The minimum inhibitory concentration (MIC) is the lowest concentration required to inhibit growth, while the minimum bactericidal concentration (MBC) is the lowest concentration to cause cell death. When used in high concentrations most biocides have more than one target site and will be biocidal; however, when used at sub-inhibitory concentrations the action may be reduced to one target and the killing activity could be lost (Denyer, 1995; Poole, 2002; Ortega Morente *et al.*, 2013). Denyer (1995) described the stages of interaction between a biocide and a bacterial cell (Figure 2.3). After its uptake by the cell the biocide migrates to the target site(s) located within the cell wall, the cytoplasmic membrane or within the cytoplasm. Membrane-active biocides

interact with proteins or phospholipids on the cell surface or within the cell wall (or outer cell membrane in Gram-negative bacteria) changing the structural integrity of the membrane and increasing its permeability (Table 2.6). The lipopolysaccharide (LPS) layer and outer membrane of Gram-negative bacteria can act as an additional permeability barrier decreasing its susceptibility to biocides compared to Gram-positive bacteria (Stickler, 2004). Still, cationic compounds such as chlorhexidine (CHA) and quaternary ammonium compounds (QACs) disrupt the outer membrane and cell wall by interacting with negatively charged polar groups of phospholipids (Gilbert and Moore, 2005). Glutaraldehyde (GTA) and ethylenediamine tetraacetic acid (EDTA) also increase the permeability of the cell wall. GTA causes irreversible cross-linking of amino groups in membrane-bound proteins resulting in fixation of the membrane (Gorman *et al.*, 1980). It is an electrophilic agent and exerts additional effects once entering the cell inhibiting enzyme activity by reacting with nucleophilic groups and causing coagulation of the cytoplasm. EDTA permeabilizes the outer membrane by causing the release of LPS. It enhances biocide activity by binding the Mg^{2+} molecules stabilizing the negative charges and cross-bridges of LPS in the membrane (Vaara, 1992; Stickler, 2004).

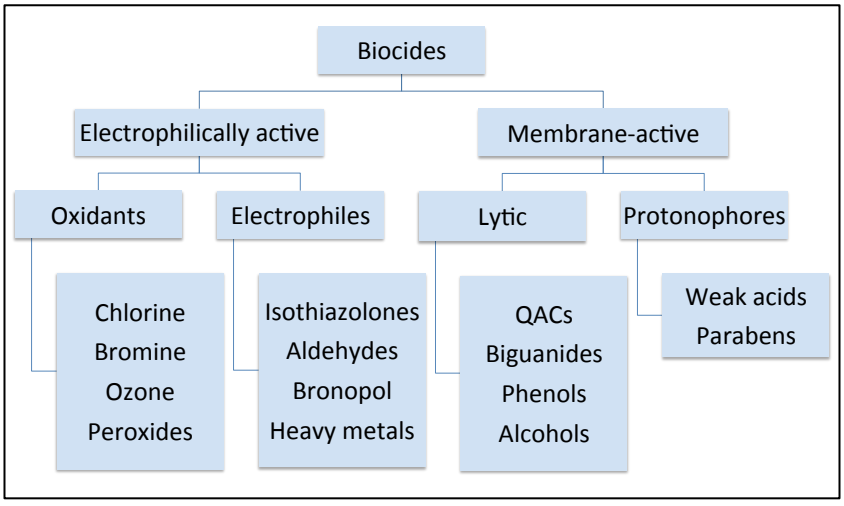


Figure 2.2 - Categorization of biocides based on mechanisms of action.
Electrophilically active biocides target nucleophilic groups within the membrane and biomolecules within the cytoplasm and inhibit cellular processes. Membrane-active agents act on lipids and proteins within the membrane to increase permeability and interfere with enzymatic activity. Quaternary Ammonium Compounds (QACs). Modified from Williams (2007).

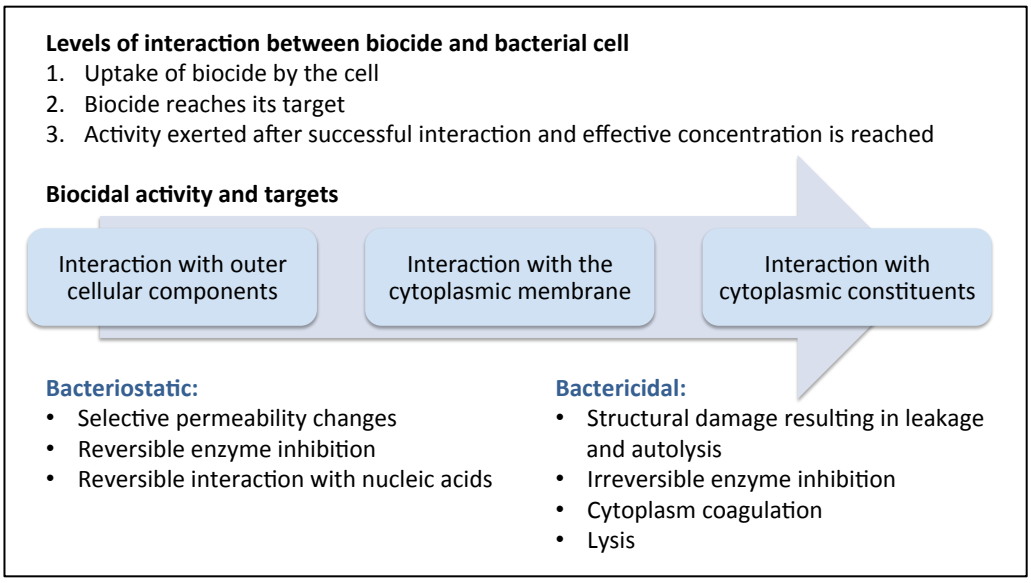


Figure 2.3 - Levels of biocidal interaction and activity in bacterial cells.
Adapted from Denyer (1995) and Maillard (2002)

Another major biocidal target site is the cytoplasmic membrane. In addition to QACs other membrane-active agents such as phenolics, biguanides, parabens, and alcohols interact with and cause disruption to the membrane and its activities (Table 2.6). Damage can result in increased permeability and leakage of intracellular components, interference with membrane-associated enzyme activity, or dissipation of the proton motive force. In each instance the disruption severely impacts metabolic activity, inhibits cellular functions in the membrane and within the cytoplasm and leads to cell death. Membrane-active biocides have a long-term effect since the interaction with their targets is generally reversible (Paulus, 2005). Electrophilically active agents including aldehydes, ethylene oxide, acridines, bronopol and other compounds containing an activated halogen atom, and isothiazolones and other substances containing an activated N-S bond, target biomolecules within the membrane and the cytoplasm. These biocides attack nucleophilic amino, thiol, amide, sulphydryl, carboxyl, and hydroxyl groups of proteins and nucleic acids. The impact within the cell is the inactivation of cellular processes caused by protein denaturation and coagulation and precipitation of biomolecules within cytoplasm (Table 2.6). After reacting with nucleophilic groups the compounds are reduced and lose their antimicrobial activity. Once isothiazolones react with thiol groups the ring structure opens and the compound loses electrophilicity (Williams, 2007). Although electrophilic biocides are fast-acting their activity dissipates over time. The effectiveness and duration of activity is dependent upon the microbial load and the concentration applied. For long-term

protection of a product a high concentration would be needed. Membrane-active biocides are preferred since they remain intact and their antimicrobial activity persists.

Table 2.6 - Target sites and mechanisms of action for different biocides

Target site	Chemical groups affected or targeted structures	Damage due to biocidal activity	Examples of biocides
Cell wall	Cross-linking of proteins; reacting with polar groups of phospholipids	Changes to structural integrity, release of cell wall components resulting in increased permeability	GTA; EDTA
Outer membrane (Gram-negative bacteria)	Polar groups of LPS and phospholipids		Cationic compounds: CHA, QACs
Cytoplasmic membrane	Phospholipids		Biguanides, QACs, detergents, alcohols
	Membrane-bound proteins and enzymes	Inhibited respiration, ATP synthesis, transport processes, enzyme activity	Alcohols, QACs, phenolics, CHA, bronopol, ISTs
	Increased permeability to protons and other ions causing acidification of the cytoplasm by	Dissipation of proton motive force, active transport inhibited, uncoupling of oxidative phosphorylation	Organic acids, parabens
Cytoplasmic constituents	Carboxyl groups	Inhibited enzyme activity	Ethylene oxide
	Thiol groups Sulphydryl groups		Heavy metals, bronopol, ISTs, hydrogen peroxide
	Amino groups		IST, formaldehyde, GTA
	Intercalation of nucleic acids	Irreversible binding	Acridines, aldehydes
	Denaturation of enzymes and proteins	Coagulation of cytoplasm	CHA, acridines, GTA, QACs, phenolics

Adapted from Maillard (2002). Glutaraldehyde (GTA); Ethylenediamine tetraacetic acid (EDTA); Chlorhexidine (CHA); Quaternary ammonium compounds (QACs); isothiazolones (ISTs).

2.3.2.3 Acquired resistance to biocides

Bacteria vary in their adaptive responses to environmental stresses like changes in temperature, pH, salinity, hydration, and nutrient deprivation. Similarly, susceptibility to antimicrobial agents can differ between organisms, between species, between strains of the same species and can change over time based on acquired or intrinsic tolerance and resistance. Acquired resistance is developed in response to repeated exposure to the antimicrobial. Adaptations to cell physiology and activity can arise through mutations or changes in endogenous gene expression. Alternatively, the acquisition of extrachromosomal elements such as plasmids or transposons can encode these changes. Common mechanisms of

resistance include target site alteration, inactivation of the antimicrobial agent, and cell wall modifications to decrease permeability or increase efflux systems. Resistance to the biocide triclosan is conferred by mutations to the *fabI* gene and its homologues in *E. coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Mycobacterium smegmatis* (Heath *et al.*, 1998; Parikh *et al.*, 2000; Heath *et al.*, 2000; McMurry *et al.*, 1999). Triclosan typically binds to the active site of the FabI enzyme impairing fatty acid biosynthesis and inhibiting cell growth (Heath *et al.*, 1999). Mutated FabI does not form this complex and provides resistance to triclosan. An example of biocide inactivation is formaldehyde dehydrogenase activity observed in *E. coli*, *Pseudomonas* sp., *Serratia marcescens, Citrobacter freundii*, and *Methylobacterium* sp. (Di Maiuta *et al.*, 2009; Kaulfers and Marquardt, 1991; Kummerle *et al.*, 1996). Resistance is achieved by the degradation of formaldehyde before it can exert irreversible effects within the cell.

Modifications to the cell wall can also decrease biocide susceptibility. Changes to the fatty acid composition, surface charge, hydrophobicity, and outer membrane protein (OMP) expression levels all facilitate resistance to QACs in *Pseudomonas aeruginosa* (Buffet-Bataillon *et al.*, 2012; Jones *et al.*, 1988; Guérin-Méchin *et al.*, 2000; Guérin-Méchin *et al.*, 1999). Biocidal targets can become inactivated, passage into the cell becomes more restricted, or there can be increases in efflux pump systems within the membrane. OMPs can play a mechanical role in maintaining membrane structure, some can interact to form porins that regulate the entry of solutes into the cell, and others may be part of efflux mechanisms. Antimicrobial resistance arising from multidrug efflux systems has been characterized in a wide range of organisms (Poole, 2004; Levy, 2002; Maseda *et al.*, 2009; Poole, 2005; Alonso-Calleja *et al.*, 2015). Increased expression levels of the OMP OprR correlated with resistance to QACs in *P. aeruginosa* (Tabata *et al.*, 2003). Transcriptomic analyses of *E. coli* and *Salmonella typhimurium* exposed to triclosan revealed increases in the expression of genes encoding components of efflux systems and their regulators in both organisms (Bailey *et al.*, 2009). Differential expression of ribosomal subunit genes and fatty acid biosynthesis genes was also observed indicating additional resistance strategies. Advances in sequencing technologies have enabled more in-depth analysis of genetic mechanisms of resistance. For instance, decreased susceptibility of biofilms to GTA is traditionally attributed to intrinsic resistance and phenotypic characteristics (discussed below). RNA-seq data from *P. fluorescens* and *P. aeruginosa* biofilms has uncovered the contribution of efflux pumps to GTA resistance (Vikram *et al.*, 2015).

2.3.2.4 Intrinsic resistance to biocides

Intrinsic resistance is conferred by a chromosomally encoded property of the organism. The outer-membrane barrier composed of LPS and porins in Gram-negative bacteria is an example of innate resistance. Another example is the cell envelope of mycobacteria that has been described as waxy due its high lipid content (Trias and Benz, 1994). Other studies have demonstrated that the bacterial growth rate and environmental conditions influence susceptibility to biocides. Cells grown in batch culture undergo physiological changes to the cell envelope as nutrients are being depleted and before the onset of stationary phase. Upon gradual depletion of magnesium in the media *P. aeruginosa* was shown to have lost its susceptibility to polymixin B and EDTA (Brown *et al.*, 1990). Sensitivity was restored after several generations in magnesium-rich media. Brown *et al.* (1990) described several physiological changes in response to nutrient deprivation:

i. Rationing of the nutrient by using different substrates and decreasing the production of cellular macromolecules containing the nutrient
ii. Modifications to the cell surface components to increase the affinity and uptake of the limiting nutrient
iii. Reduction in the growth rate to facilitate i and ii

Altering the composition of the cell wall and membranes could bring about changes in the surface charge or number of acidic residues available, phospholipid content and packing, and number of membrane proteins (Brown *et al.*, 1990). This changes the distribution and availability of antimicrobial target sites and reduces the susceptibility of the cells. Since the phenotypic adaptation is the result of environmental conditions it is considered to be a form of intrinsic resistance.

Protection against antimicrobials is also achieved within biofilms. Biofilms are composed of a dense population of cells attached to a surface. In the environment they are comprised of a consortia of microorganisms but they can also exist as a monoculture (for instance, *S. epidermis* biofilms on indwelling medical devices). A characteristic of biofilms, including multi-species communities, is cooperative metabolism where there is substrate exchange between organisms and metabolic products are distributed or removed by different community members (Davey and Toole, 2000; Stoodley *et al.*, 2002). The outer barrier of the community, the glycocalyx, is a complex extracellular matrix of excreted polymers, enzymes, metabolites and cells. It serves to protect the inner population of cells by slowing the diffusion of antimicrobial agents, adsorbing or quenching their activity, or by enzymatic degradation of the compounds (Gilbert *et al.*, 2004; Brown and Gilbert, 1993). Another characteristic of biofilms is the physiological heterogeneity of the

community. The glycocalyx and numerous layers of cells comprising the biofilm leads to nutrient, chemical, electrochemical, and gaseous gradients within the structure (Gilbert *et al.*, 2004). The primary colonizing cells at the interior have less access to key nutrients and ions, which gives rise to a reduced growth rate. They are more resistant to antimicrobials due to the differences in cell envelope composition described above. Cells along the periphery have the greatest access to nutrients and deplete them as they diffuse towards deeper layers. These cells are physiologically similar to planktonic cells with a higher growth rate and increased susceptibility to antimicrobial agents. After biocidal attack macromolecules are released during cell lysis and provide nutrients to the surviving cells. As these cells increase their metabolism and growth rate they enter a more sensitive state, succumb to antimicrobial activity, and the cycle repeats until the biofilm is destroyed. However, if the active substances are sequestered or deactivated after exerting their initial activity the biofilm has the potential to become re-established. Biofilms are highly resistant to oxidizing biocides such as halogen-releasing compounds, isothiazolones, QACs, biguanides and phenolics (Gilbert *et al.*, 2004) and pose a problem to many industries including water treatment (Rochex and Lebeault, 2007; Lazarova and Manem, 1995), cooling water systems (Purkiss, 1976; Murthy and Venkatesan, 2008), , food processing (Faille *et al.*, 2014), paper production (Vaisanen *et al.*, 1994; Desjardins and Beaulieu, 2003; Flemming *et al.*, 2013), paints (Waghela, 2014; Lindner, 2005), metal working fluids (Saha and Donofrio, 2012) and bacterial infections and health (Fujimura *et al.*, 2015; Singh *et al.*, 2000; Fux *et al.*, 2005).

Some organisms are intrinsically resistant to biocides from their ability to form endospores. Strains of *Bacillus* and *Clostridium spp.* can form a dormant cell structure to protect themselves from damage in a stressed environment. The sporulation cycle is beyond the scope of this discussion but a brief summary of key attributes of the spore structure provides insight into its protection against antimicrobial attack (Figure 2.4). The exosporium is found in many but not all bacterial spores and is composed of mostly protein. The inner and outer spore coats are also predominantly protein, have high cysteine content, and are heavily cross-linked by disulphide bridges (Leggett *et al.*, 2012). The coat layers are highly impermeable and play a critical role in maintaining resistance. The function of the underlying outer spore membrane remains unclear. The cortex and germ cell wall are both composed of peptidoglycan (PG). The germ cell wall becomes the cell wall after germination. The cortex PG is structurally different and contains a low level of peptide side chains and cross-linking. N-acetylmuramic acid is replaced by the

spore-specific residue muramic-δ-lactam (M-L), which is targeted for degradation by cortex-lytic enzymes during spore germination (Atrih *et al.*, 1998). The lipid composition of the inner membrane is similar to the vegetative cell membrane but it is more compressed and the lipids are less mobile conferring decreased permeability (Cowan *et al.*, 2004). Protein content also differs between the membranes. The spore inner membrane contains germinant receptors and putative dipicolinic acid (DPA) channels. DPA is thought to enhance spore resistance to wet heat by reducing the core water content and also help protect spore DNA against damage and antimicrobial attack (Setlow *et al.*, 2006). The dehydrated spore core contains DNA, RNA, ribosomes and enzymes. Its low water content contributes to enzymatic dormancy and protects the core from heat and chemical treatment (Setlow *et al.*, 2006). The complex spore structure provides effective resistance to antimicrobial agents. Some substances are sporistatic preventing germination and outgrowth but are not sporicidal such as phenols, organic acids and esters, QACs, biguanides, alcohols, and organomercurials (Russell, 1998). Chlorine-releasing agents, ethylene oxide, aldehydes, hydrogen peroxide, iodine compounds and peroxy acids are sporistatic and sporicidal at very high concentrations and long contact times (Russell, 1998).

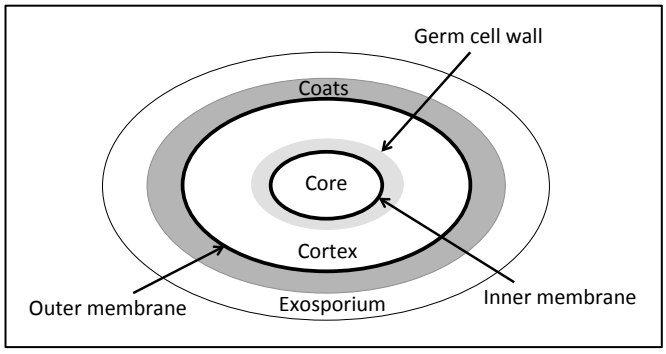


Figure 2.4 - Bacterial spore structure.
The spore core containing DNA, RNA, ribosomes and enzymes is highly resistant to heat and most antimicrobials due to the protective structural layers of the outer and inner spore coats, the cortex, and inner membrane. Structures are not to scale. Adapted from Leggett et al. (2012)

Non-sporeforming organisms also form cells exhibiting a dormant phenotype. Commonly observed examples are human pathogens causing chronic or latent infections. *M. tuberculosis* can adopt a low-replicative state after infecting the host and lead to latent tuberculosis. A long antibiotic treatment course is required to manage chronic infections and prevent relapses from dormant cells that are desensitized to antibiotics and have evaded the immune system (Rittershaus *et*

al., 2013). *S. aureus, P. aeruginosa* and *E. coli* have also demonstrated tolerance to antibiotics by forming populations of these specialized survivor cells known as persisters (Keren *et al.*, 2004). The phenomenon of bacterial persistence was first described in the 1940s and was used to explain the recurrence of Staphylococcal infections despite high doses of antibiotics (Hobby *et al.*, 1942). Bigger (1944) proposed the existence of dormant, non-dividing cells that were capable of surviving antibiotic treatment and responsible for chronic infections. Dormant cells have also been detected in ecosystems in soil, marine water, fresh water, activated sludge and the human gut (Lennon and Jones, 2011). *P. fluorescens* and *Vibrio vulnificus* have been shown to enter dormant states increasing their long-term survival in soil and cold aquatic environments respectively (Dworkin and Shah, 2010). Lennon and Jones (2011) compared data from various characterization studies of populations in different environments. Cellular activity was assessed by fluorescence *in situ* hybridization (FISH) using general eubacterial probes or by the uptake and accumulation of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Soil was found to have the highest proportion of inactive cells (83%) followed by fresh water (45%) and marine water (38%). The authors suggested that seed banks of dormant cells act as a reservoir for the organism during periods of environmental stress. This prolongs the persistence of a population and enables its resuscitation when favourable conditions return. Similar to the induction of sporulation, dormancy can be initiated in response to fluctuations in temperature, pH, osmotic pressure, nutrient and resource limitations and toxic chemical concentrations. However, dormant cells differ from spores since they display nominal metabolic activity, maintain membrane potential, and do not undergo gross morphological changes.

Dormant cells can also be formed spontaneously. Lewis (2007) examined persister cell formation in *E. coli* grown in culture and observed an increase in persisters during the mid-exponential phase. The spontaneous generation of persisters avoids the cellular energy costs associated with sensing environmental conditions and facilitates a rapid protective response when the environment is perturbed (Lennon and Jones, 2011). Some phenotypic characteristics of dormant cells include increased accumulation of energy storage compounds such as fatty acids, a reduction in metabolism and enzyme activity, modifications to the cell wall, and reduced synthesis of macromolecules (Rittershaus *et al.*, 2013). This reduction in DNA, RNA, and protein content and alterations to the quantity and composition of lipids in the membrane leads to a reduced cell volume (Lennon and Jones, 2011). Typical antimicrobial targets including membrane and intracellular components are inactivated facilitating increased tolerance to antimicrobials. However, persistence

is a transient phenotype and a population regrown from persisters becomes sensitive to antibiotics (Gefen and Balaban, 2009).

Exit from dormancy can be triggered by growth-promoting environmental cues as observed in spores; for instance, spore germination in *Bacillus* spp. is stimulated upon detection of amino acids or PG-derived muropeptides in the environment (Dworkin and Shah, 2010; Moir *et al.*, 2002). In non-sporulating bacteria the scout model has been proposed whereby individual dormant cells awaken stochastically to assess environmental conditions (Epstein, 2009b). If adverse conditions still persist the awakened scout cell dies and is subsequently replaced by new scouts. If conditions are favourable it can give rise to a new population and in some species the scout may produce growth-inducing signals (Epstein, 2009a). Dormant cells and scouts are genetically similar to active cells. While dormant cells exhibit minimal metabolic activity, scouts are hypothesized to awaken after a random change in expression or repression of an unidentified master regulatory gene (Buerger *et al.*, 2012). The scout model divides a clonal population into dormant or active phenotypes and one's dominance over the other depends on the detected environmental conditions. Buerger et al. (2012) evaluated awakening of microorganisms in soil and marine sediment samples and laboratory cultures of *E. coli* and *M. smegmatis* enriched for dormant cells or spores after exposure to antibiotics. The observations from the cultivation experiments were that most of the cells in environmental samples were viable but dormant, and different rates of colony formation by organisms of the same species depicted stochastic awakening. Single-cell experiments of the marine sediment samples and *E. coli* and *M. smegmatis* dormant cell-enriched cultures were performed over several months. Dormant cells (of the same species) re-initiated growth at different time points despite exposure to the same growth conditions. When isolates were sub-cultured the observed heterogeneity in the growth rates was lost. These findings supported the theory that cells awaken stochastically and also suggested the process is nutrient-independent. While this has implications for the study and treatment of persistent and reactivated latent infections, it should also influence our interpretation of cultivation experiments when characterizing microbial populations.

2.3.3 Monitoring contamination and microbial communities

Close monitoring of CCPs is an integral part of the HACCP plan in food manufacturing. End-product testing is required to ensure the quality, safety and conformance of products prior to their release to the market, but it cannot pinpoint

the source of a contamination problem. In-process testing at CCPs is more informative and can facilitate swifter detection and correction of failures. There are a range of techniques for assessing microbial load, community composition, and physiological activity. They possess different strengths and weaknesses but ultimately regulations and costs may influence their implementation on an industrial scale.

2.3.3.1 Culture based methods

Conventional culture methods remain the gold standard for enumerating the total viable count (TVC) of microorganisms within a sample matrix, typically expressed as colony forming units per milliliter ($cfu \cdot mL^{-1}$). The FDA's Bacteriological Analytical Manual outlines laboratory procedures for the analysis of bioburden in foods and cosmetics (available online at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm). Detection of pathogens including *L. monocytogenes*, *S. aureus*, *Salmonella*, *Coliforms*, *E. coli*, *Campylobacter jejuni*, *B. cereus*, and yeasts and moulds in products is still widely determined by culture. The main advantages of these methods are their low cost and ease of use but regulators and the industry at large also tout their reliability and accuracy. A century of cultivation research, experience, and optimization have verified the suitability and limits of detection of these methods. Furthermore, pathogenic organisms have been well studied in cultivation experiments and regulatory agencies have used this knowledge to set microbial limits according to these methods to ensure consumer safety. Yet, the field of microbiology has evolved and embraced additional techniques and technologies for microbial detection. Once widely used as the sole method for characterizing microbial communities, assessing the effectiveness of antimicrobial agents, and estimating microbial load, the application of and reliance on culture methods in research has diminished.

Microbial ecologists had long remarked on the discrepancy between TVCs of samples cultured on plates and direct counts by microscopy. Staley and Konopka (1985) re-introduced the concept of the 'great plate count anomaly' when they observed that only 0.1-1% of the total bacteria in lake samples was recovered on plates. Amann *et al*. (1995) perpetuated this finding while promoting 16S rRNA gene analyses for uncovering microbial community composition and abundance. This lack of recovery and lack of diversity seen in culture has been attributed to the inadequacy of media and culture conditions, slow growth rate of some organisms,

an abundance of cells within the hypothetical viable but not culturable (VBNC) state, or low prevalence of organisms within a population.

Certain bacteria may have strict requirements for growth needing specific concentrations of nutrients, oxygen level, pH conditions, or a narrow incubation temperature range. Culturing on a variety of growth media, employing longer incubation times, and varying inoculum size all impacted positively on viable count and colony formation of rare species in soil samples (Davis *et al.*, 2005). High cultivation efficiencies were achieved in tidal sediment samples by using a diversity of growth substrates and culture conditions (Köpke *et al.*, 2005). Greater diversity can also be recovered by simulating the natural environment. Culturing samples in diffusion chambers or microbial traps within their natural habitat allows the passage of important unidentified signalling compounds or metabolites present in the environment. These could be generated by other community members and may be required for growth of some species. This method aided in the isolation of previously uncultured species from marine sediment (Kaeberlein *et al.*, 2002; Epstein, 2013).

The VBNC state was proposed to explain the phenomenon of organisms losing culturability in response to stress (Xu *et al.*, 1982). The cells are considered to be viable but dormant and unable to grow under routine culturing procedures. Resuscitation occurs once the stress is removed and the environmental conditions are favourable for growth (Oliver, 2010; McDougald *et al.*, 1998). A number of bacteria have been shown to enter this state (Oliver, 2005; Oliver, 2010). There were some who challenged the concept believing instead that VBNCs were injured or dead cells (Kell *et al.*, 1998; Bogosian and Bourneuf, 2001). Additional doubts arose from the presence of a residual low level of culturable cells despite the supposed dormant state (Kell *et al.*, 1998) and the inability to resuscitate non-culturable cells (Bogosian and Bourneuf, 2001). The scout model described above has been applied to explain observations of the VBNC concept. The large pool of dormant cells is indeed in the VBNC state and instances where minimal growth was observed were from the continuous generation of scouts (Buerger *et al.*, 2012). The scout model also has implications for perceived slow-growing organisms. The late appearance of slow-growers in culture could be due to their late revival from dormancy as opposed to an inherently low growth rate (Buerger *et al.*, 2012).

Poor or slow recovery in culture can be a reflection of low abundance but it can also relate to the physiological status of cells (Hattori *et al.*, 1997). If there are a higher proportion of quiescent cells than active cells then less growth is observed and there is an underestimation of abundance. If an organism is in low abundance

and dormant there is even less likelihood of its detection in culture. Under the scout model less abundant species would have few or no scouts present and would appear to be uncultivable (Epstein, 2013). Schmidt and Konopka (2009) proposed two strategies for accessing these organisms. One approach is the brute force strategy of increasing sampling and increasing cultivation efforts. For instance, high-throughput extinction culturing by diluting samples with low-nutrient media or sea water enabled the recovery of previously uncultured marine *Proteobacteria* clades (Connon and Giovannoni, 2002; Rappé *et al.*, 2002). The other approach entails directly targeting the organisms in question. This could involve longer incubation times to wait for dormant cell awakening or fractionating a sample to increase the probability of detecting rare organisms. Davis *et al*. (2005) observed increases in viable counts with increasing incubation times up to 16 weeks.

Microbial ecologists and industrial microbiologists have different requirements and expectations of microbiological methods. For industry the most essential information required from routine microbial monitoring is an estimation of viable count and the presence or absence of pathogens. In the production of non-sterile products the total cell count including the proportion of dormant cells is generally viewed as unnecessary (unless the organisms are pathogenic). Therefore, traditional culture methods remain to be a suitable technique for assessing microbial load of non-pathogenic background organisms. Still, some instances may necessitate more sensitive and more specific microbiological information, for example in cultivation experiments employed for testing the effectiveness of surface disinfectants (Lambert, 2004) and preservatives for food and cosmetic products (Russell, 2003). In-use antimicrobials being evaluated for protecting foods and cosmetics undergo multiple challenge tests. Regulatory agencies have specified microbial load or pathogen detection limits for some products (Hiom, 2004; Sutton and Jimenez, 2012). Antimicrobial efficacy is gauged by log-reductions in bioburden in a given time period. The item or formulation being preserved is treated with different concentrations of the antimicrobial agent and inoculated with designated test organisms. After prescribed incubation times samples are collected, the preservative activity is neutralized (by dilution or quenching) and the sample is cultured on solid media (Russell, 2003). The viable count is determined and the MIC and MBC are identified. Since sub-lethally injured cells do not readily recover on growth media (Mackey, 2000) the frequency of colony formation does not reflect the true viable count of the population. The recorded MIC and MBC indicate the concentrations at which the damage to the cells was sufficient to inhibit growth on media, however the situation *in situ* could be

different. Injured cells may be able to repair and recover within the product environment (in the absence of additional stress) and there is the risk of underestimating the MIC. The presence of a dormant population should also influence the interpretation of colony visualization in culture, which also has implications for MIC determination. Common misinterpretations of plate counts are described in Table 2.7. The absence of viability has been characterized as the inability to divide and form colonies under any tested culture condition (Kaprelyants *et al.*, 1993). Although this accurately defines non-viable/dead cells, the lack of visible growth on a plate does not necessarily indicate the absence of viable cells within the tested sample.

Table 2.7 - Interpretations of colony visualization

Observed result	General interpretation	Alternative explanations
A colony was formed	A viable cell gave rise to the colony	The colony was formed from the growth of at least one viable cell
There were no visible colonies	There were no viable cells in the sample	i. The culture conditions (media, incubation time or temperature) were not suitable for growth of the organism(s) ii. The cells were injured or stressed prior to cultivation and unable to grow/recover under the specified conditions iii. The plates were incubated for an insufficient time to allow observable colony development of slower growing organisms or dormant cells iv. The population density of the plated sample was too low - impaired cell-cell communication with the absence of metabolites or signaling molecules generated within the community

Adapted from Davey (2011)

From an industrial standpoint, another key disadvantage of culturing is the length of time to result. The challenge testing process is time-consuming, costly, and laborious (Russell, 2003). Standard pathogen testing of manufactured products is also labour-intensive and can take several weeks. Initial results for most organisms may take 2-3 days but identification and confirmation of a positive result could take 7-10 days (Velusamy *et al.*, 2010). Throughout a manufacturing process CCPs are monitored in real-time. Culture methods are neither sensitive enough nor capable of delivering results in that timeframe. As previously mentioned, critical limits of CCPs are often quantifiable measurements that can be monitored in real-time. Although samples may still be taken for microbial analysis at the CCP, the process control limit (e.g. temperature) is used to indicate the risk or likelihood of contamination at that point in the process. If the limit is outside of the range known

to minimize microbial load, action can be taken immediately to correct or treat the product. The ultimate CCP measurement would provide bioburden estimation in-process; instead, actual microbiological data is typically only consulted in finished product testing. For many years there has been a push from industry to investigate rapid microbiological methods (RMMs). They have the potential to help improve, strengthen, and validate HACCP systems, increase process efficiency, decrease product and batch-release times, and improve the safety and microbial quality of finished products.

2.3.3.2 Rapid microbiological methods (RMMs)

Rapid microbiological methods offer industry and academia a more sensitive, accurate, and specific alternative to traditional culture-based methods. There are a wide variety of technologies available commercially, in development, or modified and designed for individual laboratory use. To be considered "rapid" they must provide microbiological information in a shorter timeframe than cultivation methods. Before they can be implemented in routine microbial quality testing, RMMs require approval from the relevant regulators. (Some industrial products and sectors are less restricted and do not require approval). The first step in achieving this aim is passing the ISO validation process (ISO 16140:2009). This guideline describes the process for evaluating a new microbiology detection method. Identical samples are concurrently tested using reference culture methods and the alternative technique. Reference culture methods are those that are traditionally used (as prescribed by regulators) or those that are internationally recognized. ISO has a vast range of reference microbiological procedures for specific pathogens, food products, meats, dairy, water, textiles, the environment etc. The internationally recognized Association of Analytical Communities International (AOAC) is an independent group of experts from industry, academia, and governments that evaluates and publishes standardized analytical methods for chemistry and microbiology in the *Journal of AOAC International*. Their Official Methods are recognized as standard methods by several regulatory agencies including the FDA. Another requirement of the validation process is inter-laboratory testing, which serves to prove the precision, robustness and reproducibility of the results (Jasson *et al.*, 2010). A common route for validation is through the AOAC.

RMMs are broadly classified into nucleic acid-based methods, biosensor-based, immunological-based, growth-based, and viability-based technologies.

2.3.3.2.1 Nucleic acid-based technologies

Nucleic-acid based methods detect specific DNA or RNA sequences of the targeted organism(s). For pathogen testing, oligonucleotide probes are designed targeting virulence factors such as *Salmonella* invasion protein A or listeriolysin O produced by *L. monocytogenes*. For overall microbial community analyses, amplification and sequencing of 16S rRNA genes and other housekeeping genes is used for identification. Multiplex-PCR (mPCR) facilitates simultaneously targeting of several genes (or several organisms), which is both less time-consuming and more cost-effective. Quantitative or real-time PCR has the advantage of not requiring gel electrophoresis to visualize PCR products. During amplification, fluorescently-labelled probes or intercalating dyes are incorporated into amplicons and their formation can be monitored as the reaction progresses. Fluorescence intensity is proportional to the amount of a PCR product present, hence the quantitative aspect of the method. Real-time mPCR assays have also been widely developed and applied in industry and academia. For instance, Hu *et al*. (2014) developed a real-time mPCR assay that allowed the detection of eight foodborne pathogens. An overview of additional advantages and disadvantages of traditional PCR-based methods is provided in Table 2.7.

The high sensitivity and specificity of PCR has allowed it to become one of the most accepted RMMs by regulators. ISO has published a protocol for the real-time PCR-based detection of Shiga toxin-producing *E. coli* in environmental samples from food production/handling areas and in foods for human and animal consumption (ISO/TR 13136:2012). The guideline ISO 22119:2010 outlines the minimal requirements for any real-time PCR assay used for detecting pathogens. A number of these commercial assays have been successfully validated by the AOAC. The US Food Safety and Inspection Service has approved and/or directed the use of several PCR-based assays for the screening of carcasses, meat, poultry, eggs, fish and shellfish and fruits and vegetables for pathogens (*L. monocytogenes*, enterotoxigenic *E. coli*, *V. vulnificus*, *V. cholerae, Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, Avian Influenza, Hepatitis A, and Norwalk viruses) (US FDA, 2015). These assays are also often a requirement for sterility testing in the production and manufacturing of pharmaceutical products. In some instances the FDA has also endorsed the use of a particular instrument on which to carry out analyses (e.g. BAX® Detection System (DuPont Qualicon)).

In addition to continually optimizing PCR methods, other techniques for amplifications have also been developed. Nucleic acid sequence-based amplification (NASBA) is a method for isothermal amplification of DNA or RNA. It

has gained interest in recent years due to its ability to selectively amplify mRNA from within a background of genomic DNA (Zhao *et al.*, 2014). Real-time NASBA delivers the same advantages of real-time PCR, but in this instance mRNA is targeted for amplification and the fluorescence intensity measured is from viable cells alone. NASBA kits are commercially available from Life Sciences Advanced Technologies, bioMérieux, GenProbe, and KIT Biomedical Research and have been successfully used to detect multiple pathogens (Gracias and McKillip, 2007)(Table 2.9). Another amplification method called loop-mediated isothermal amplification (LAMP) was developed by Notomi *et al*. (2000) and is based on auto-cycling strand displacement DNA synthesis. The reaction utilizes 4-6 primers targeting 6-8 sequences within the target gene and the unique mechanism of amplification generates many different sizes of stem loop DNAs containing inverted repeats of the targeted sequence (Zhao *et al.*, 2014). This method benefits from increased specificity and a greater yield of amplification products. Real-time LAMP has also been successfully tested. Commercial LAMP kits are available for detecting many foodborne pathogens (Table 2.9) and for diagnostics of infectious diseases (Mori and Notomi, 2009; Niessen *et al.*, 2013).

Microarrays are another example of a nucleic acid-based technology. Oligonucleotide probes of 25-80 bp targeting specific genes for identifying pathogens are coated onto a glass slide or chip. Fluorescently-labelled nucleic acid fragments (of DNA, mRNA, or cDNA) from the sample are allowed to hybridize to the corresponding target sequences on the chip. A fluorescence signal is produced upon successful binding and the intensity measured is proportional to the amount of nucleic acid present (Law *et al.*, 2015). Microarrays have been used in research to identify pathogens by virulence factors (Sergeev *et al.*, 2004) and the 16S rRNA gene (Wang *et al.*, 2007). The design of most commercial microarrays is optimized for gene expression studies, which require a high-density of probes. For food applications where only a few pathogens will be tested, lower density arrays are more suitable and must be custom fabricated (Rasooly and Herold, 2008).

2.3.3.2.2 Biosensor-based technologies

A biosensor contains a bioreceptor and transducer component. The bioreceptor comprises a biological material such as antibodies or antigens, enzymes, nucleic acids, cellular receptors or biologically-derived materials (e.g. recombinant antibodies, engineered proteins) (Zhao *et al.*, 2014). Detection is based on binding of a target analyte within the sample with the bioreceptor. The transducer converts this interaction into an electrical signal that can be optical, electrochemical, or mass-

based (Law *et al.*, 2015). The signal is amplified, processed by a signal processor, and the data is analysed, displayed and stored. Optical-based biosensors exploit chemical properties of the analyte-bioreceptor reaction including light absorption, reflection, refraction, luminescence, fluorescence, and Raman scattering. Several commercially available biosensor-based RMMs utilize surface plasmon resonance (SPR) for microbial detection (Table 2.9). Interactions between bioreceptors immobilized on a precious metal film and the analyte generate a change in the refractive index of the metal surface after exposure to visible or near-infrared radiation. The extent of the shift in resonance corresponds to the concentration of bound pathogens (Long *et al.*, 2013). Electrochemical biosensors detect physicochemical changes that result from the interaction between the bioreceptor and its target. Those that detect changes in current are amperometric, potentiometric sensors detect alterations in voltage, conductometric devices measure changes in electrical conduction, and impedimetric sensors detect shifts in impedance (Grieshaber *et al.*, 2008). In general these biosensors are not as sensitive as optical sensors and have not received the same support for commercialization (Table 2.9). In mass-based biosensors bioreceptors (typically antibodies) are immobilized on a piezoelectric crystal that vibrates at a specific frequency when an electrical signal is applied. The binding of the target antigens to the antibodies increases the mass at the crystal surface and leads to a shift in its vibrational frequency. Although some success has been achieved in pathogen detection this type of biosensor is not widely adopted (Velusamy *et al.*, 2010) (Table 2.9).

2.3.3.2.3 Immunological-based technologies

Immunological assays are based on the binding of a specific antibody to its target antigen. Some biosensors work off this principle. The sensitivity and accuracy of these methods are dependent on the specificity of this interaction. An example of a widely commercialized and used assay is enzyme-linked immunosorbent assay (ELISA). The sandwich ELISA is often available in microtiter plate format for high-throughput analyses. The primary antibody used to target the desired antigen (bacterial toxin or cell surface protein) is immobilized on the walls of each well. The sample is added and the antigens are left to bind the affixed antibodies. After this interaction an enzyme-conjugated secondary antibody, also specific for the antigen, is added and a sandwich complex is formed. Antigen binding is detected by the addition of a colourless substrate that is converted to a detectable coloured form by the presence of the enzyme. There are alternative formats of this assay where a

second antibody is added and allowed to bind the antigen. The enzyme-labelled antibody targets this second antibody instead of the antigen, which serves to amplify the overall level of detection. A wide range of enzymes can be used but horseradish peroxidase, alkaline phosphatase and beta-galactosidase are the most common (Law *et al.*, 2015). The method requires several washing steps after each stage to reduce non-specific binding and false positive results. Several robotized automated systems are available on the market to facilitate high-volume testing, reduce the level of user-input required, and decrease the time to result. These are held to ISO standards and must be validated as an alternative microbiological method for the analysis of environmental and veterinary samples, and foodstuffs for human and animal consumption (ISO 16140:2009). Newer assays utilize a more sensitive variation of ELISA based on the generation and detection of fluorescent products: enzyme-linked fluorescent immunoassay (ELFA). The VITEK immunodiagnostic assay system (VIDAS) sold by bioMérieux uses ELFA to detect *Listeria* spp., *Campylobacter* spp., *E. coli* O157, *Staphylococcal* enterotoxins A to E and *Salmonella* spp. (bioMerieux SA, 2015c) (Table 2.9).

Lateral flow immunoassay provides a cheaper, simpler and more reliable alternative to ELISA (Law *et al.*, 2015). The device is in the form of a dipstick or immunochromatographic strip and is comprised of four sections containing different components of the assay. The sample fluid is loaded and migrates through each section by capillary action. In the conjugate pad the sample is mixed with a coloured reagent, which is typically an antibody labelled by gold particles or colloidal latex. The fluid flows through the nitrocellulose membrane section composed of distinct lines/zones of immobilized antibody. The presence or absence of the targeted analyte within the sample determines the binding location of the coloured reagent. A range of pathogens have been tested using this method including *Salmonella* (Leem *et al.*, 2014) and *Staphylococcal* enterotoxin B (Rong-Hwa *et al.*, 2010). Immunochromatographic strips have been manufactured by several companies for the detection of *Listeria* spp., *Salmonella* spp., and *E. coli* (Table 2.9).

2.3.3.2.4 Growth-based technologies

Growth-based methods provide quantitative information about the bacteria present and are not used for species identification. Monitoring surfaces and air cleanliness and bioburden levels in a production plant is an essential part of HACCP. One of the most rapid and extensively used RMMs for assessing plant hygiene relies on the detection of Adenosine triphosphate (ATP). The assay is

based on the ATP luminescent reaction in fireflies. In the presence of the luciferase enzyme, Mg^{2+}, and O_2, ATP within the sample drives the oxidation of luciferin to oxyluciferin and the release of light. The emitted light is measured by luminometer and expressed as relative light units (RLU). The preparation method has been adapted to many sample types. Some platforms require samples to be collected by swabs while others use membrane filtration. Swab devices provide results within minutes but filter systems require incubation for a minimum of 24 hours. The presence of non-microbial ATP can increase background bioluminescence and decrease the reliability of the assay (Bottari *et al.*, 2015). Several systems have included reagents and steps in the protocols to remove non-bacterial cells and other inhibitory substances in the sample matrix. The pH of the sample, the presence of proteins or debris, and the physiological state of the cells can also influence measurements. Omidbakhsh *et al*. (2014) observed that different surface disinfectants can have a quenching or enhancement effect on RLU readings. Nonetheless, there has been good overall acceptance of ATP-bioluminescence for estimating cell viability and microbial load. A large number of products are on the market from companies like Neogen, 3M, EMD Millipore (Table 2.9).

Another method of assessing bioburden level is by monitoring changes in CO_2 production of samples grown in culture. Thorpe *et al*. (1990) developed an automated detection system called BacT/ALERT that is currently sold by bioMérieux SA. The system includes the instrumentation and proprietary complex growth medium in specialized bottles. A CO_2 sensor is located at the bottom of each bottle and separated from the broth by a semipermeable membrane. The membrane is freely permeable to CO_2, nearly impermeable to water, and impermeable to most ions and components in the media (Thorpe *et al.*, 1990). The bottles are inoculated with the sample and incubated within the instrument. CO_2 is produced during microbial growth and it diffuses across the membrane and dissolves in water producing hydrogen ions. Increasing CO_2 leads to more free hydrogen ions to interact with the sensor and cause a decrease in pH. The sensor colour changes from blue/dark green to lighter-green/yellow as the sensor environment becomes more acidic. Detectors located underneath each bottle monitor changes in reflectance units. The limit of detection is higher than other methods 10-50 $cfu \cdot mL^{-1}$ (Parveen *et al.*, 2011; Moldenhauer, 2008) but the incubation step extends the assay time to 24-72 hours (bioMerieux SA, 2015a) (Table 2.9). The BACTEC FX system from Becton Dickinson is based on the same principle but detects changes in fluorescence. BacT/ALERT has been evaluated for sterility testing and pathogen detection in the production and manufacturing of vaccines and biologics (Parveen *et*

al., 2011) and both systems are used for diagnostic analysis of microorganisms in the blood. Although there has yet to be major interest from the food industry, the new BacT/ALERT 3D system is marketed for monitoring bioburden in low and high acid food and beverage products (bioMerieux SA, 2015a).

2.3.3.2.5 Viability-based technologies

Viability assays make use of fluorescent dyes to distinguish between viable and non-viable cells within a sample. A diversity of fluorescent probes is available that target different proteins or biomolecules and can provide physiological information about the cell (refer to Table 2.8). Furthermore, fluorescently labelled antibodies or nucleic acid probes can be made or purchased to target specific microbial species. Cells stained with fluorescent probes can be analyzed by several methods, the simplest being microscopy. Staining with a membrane permeant dye targeting nucleic acids allows the visualization of all cells. Membrane impermeant dyes such as propidium iodide are excluded from live cells and only enter dead or injured cells (with damaged membranes). Co-staining with these two probes facilitates the identification of live, injured, and dead cells within the sample. Although manual microscopic analysis is relatively simple and fast it is not suitable for large numbers of samples. Image analysis software is available to automate the scoring of live/dead cells but microscopy is still not practical on an industrial scale.

An alternative method that is more rapid and can accommodate staining with a combination of probes is flow cytometry. This method quantitatively measures optical characteristics of cells as they encounter a beam of light and emit a pulse of fluorescence and scattered light. Detectors transform these signals into electrical signals to be processed and analyzed. Subpopulations of cells can be discriminated from each other by the use of multiple fluorescent probes. For instance, viability can be measured by staining with nucleic acid dyes, and probes for measuring metabolic activity and membrane potential. The LIVE/DEAD BacLight kit from Molecular Probes (Thermo Fisher Scientific) is available commercially for use in research. Specialized flow cytometers for bacterial testing are also available on the market. CHEMUNEX® (bioMérieux) and BD FACSMicroCount™ System (Becton Dickinson) offer fully automated units that can process 15-20 samples per day (bioMerieux SA, 2015b; BD, 2015). The general limit of detection is 10^2 cfu·mL^{-1} (Table 2.9). Certain sample types may require enrichment prior to staining and measurement, while others such as water can be processed and analysed immediately. These systems are mainly used for non-specific viable microbial assessment, but the newer RAPID-B (Vivione Biosystems) has been used for the

detection of viable *E. coli* O157 in 15 different food products (Wilkes *et al.*, 2012). The authors purified their own polyclonal antibodies targeting multiple epitopes on the cell.

Table 2.8 - Selected fluorescent dyes and their targets

Dye compound	Physiological target
Propidium iodide	DNA in dead cells; membrane-impermeant dye, staining indicates disrupted membrane integrity
SYTO dyes (e.g. SYTO 9,13,62)	DNA in all cells
4'6'-diamidino-2-phenylindole (DAPI)	DNA in all cells
Carbocyanine dyes (e.g. 3,3'-dihexyloxacarbocyanine, diOC6(3); 3,3-dipropylthiadicarbocyanine iodide; diSC_3(5))	Negative charges within the membrane, particularly the cytoplasmic side of the inner membrane; indicates the presence of a membrane potential and viability
Ethidium bromide	DNA; staining signifies little or no efflux pump activity
Rhodamine 123	Negative charges within the membrane; informs on the status of membrane potential and efflux pump activity
5-cyano-2,3- ditolyl tetrazolium chloride (CTC)	Reduced to a fluorescent product upon entering the cell; indicative of dehydrogenase activity (Joux and Lebaron, 2000)
Carboxyfluorescein diacetate (CFDA)	Cleaved by non-specific esterases to a fluorescent product within the cells; measure of esterase activity (Joux and Lebaron, 2000)

CellFacts II (CFII) (CellFacts2014 Ltd.) is another quantitative cell viability system. This technology combines electrical flow impedance and fluorescent staining to measure bioburden levels within a sample. Isolated cells are stained with the membrane permeant SYTO 62 and diSC_3(5) dyes (Table 2.8). Cells possessing a strong membrane potential will accumulate more diSC_3(5) and the relative fluorescence for those particles will be higher. Unlike the flow cytometry systems described above, CFII does not require an enrichment step. The instrument has been successfully applied in measuring the microbial load in powdered milk, fruit juices, soluble oil, paints, pigments, and slurries (CellFacts2014 Ltd., 2015). This RMM technology was transferred to industry and evaluated as an alternative method to culture dip slides for monitoring contamination in calcium carbonate slurries (discussed in Chapter 3).

2.3.3.2.6 Enumeration and identification methods based on culturing

A number of modified culture methods have been classified as rapid methods after being streamlined, miniaturized and automated to reduce assay time. Quantitative information about bioburden can be achieved by culturing on dip slides, 3M™ PetriFilm™ (3M), or by most probable number (MPN) culture systems such as

TEMPO® (bioMérieux). Samples grown on dip slides and PetriFilm are incubated on solid media/film and the user detects colony formation visually. MPN is based on the degree of growth detected from serial dilutions of a sample when cultured in liquid media. The TEMPO system includes a card device for incubating the diluted samples. Growth is detected on the TEMPO Reader (bioMérieux) and based on changes in the concentration of a fluorescent pH indicator in the media. These methods can provide results within 24-48 hours; however, they have the same deficiencies of underestimating microbial load. Damaged cells, dormant cells, and organisms not capable of growing under the defined culture conditions will not be recovered in the short incubation time and inadequate environment.

Rapid methods have also been developed for species identification after culture enrichment. These replace traditional means of identification of culturing on different selective media, visualizing colony morphology by microscopy or large-scale biochemical tests. Miniaturization and automation has helped reduce the time to result and enhance the sensitivity of detection. Many growth-based biochemical assays are available on the market and can provide species identification of a pure culture within 24 hours. The test format can be in the form of a disk, strip, or microtiter plate with individual compartments/wells containing different dehydrated growth substrates and colourless dyes. A cell suspension is made from a single colony or pure culture and applied to each well and incubated. Substrate utilization differs between species and the pattern of colour development or turbidity on the disk, strip or plate allows for identification by reference database. Common commercial biochemical kits are API® (bioMérieux), and VITEK® 2 (bioMérieux), and MicroLog® (Biolog). The API and VITEK kits have been approved as official species identification methods by the AOAC and FDA.

Table 2.9 - Comparison of RMMs for the identification of pathogens or detection of microbial growth

Detection method	Detection limit	Advantages	Limitations	Assay time	Examples of products on the market	Additional information
Nucleic acid						
PCR • Simple • Multiplex • Real-time	10^2-10^4 cfu · mL^{-1}	• High sensitivity and specificity • Potential for the detection of multiple pathogens • Process automation available	• May or may not require enrichment • Requires DNA isolation • Difficulty distinguishing between viable and non-viable cells (unless viable cell dye exclusion approached used e.g. propidium monoazide - Chapter 4)) • Prone to PCR inhibitors • Higher cost • Requires skilled users	3-30 hours (Dependent on requirement for enrichment)	Food PCR Testing kits and automated units: • QIAGEN mericon™ • BAX® Detection System (DuPont Qualicon) • iQ-Check® Prep Solution (Biorad) • GenomeLab GeXP Genetic Analysis System (AB Sciex) (Zhou *et al.*, 2013) • GeneDisc® Rapid Microbiology System (Pall Corporation) • ADIAFOOD® (AES Chemunex)	• Detection of toxin-related genes • Pathogens successfully detected: *L. monocytogenes*, *E. coli* 0157:H7, *C. jejuni*, *Salmonella* spp., *Shigella* spp., *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* (Zhou *et al.*, 2013; Hu *et al.*, 2014) • Can also identify bacteria by sequencing of 16S rRNA gene or housekeeping genes
Nucleic acid-sequence based amplification (NASBA)	10-10^3 cfu · mL^{-1}	• Thermal cycler not required • Lower cost • Able to detect viable cells by mRNA amplification	• Requires viable cells • Challenges related to handling RNA	3 hours (Fykse *et al.*, 2007)	• Nuclisens EasyQ® Basic Kit (bioMérieux)	• Pathogens successfully detected by NASBA: *Salmonella enterica*, *V. cholerae*, *S. aureus*, *Campylobacter jejuni*, and *E. coli* (Fykse *et al.*, 2007; Nadal *et al.*, 2007)

Loop-mediated isothermal amplification (LAMP)	5-30 cfu per reaction, or 10^2-10^5 cfu · mL^{-1} (Niessen *et al.*, 2013)	• Thermal cycler not required • Lower cost	• Complicated primer design • Unable to detect unknown/unsequenced targets • Requires skilled personnel	1-3 hours	• Loopamp DNA amplification kit (Eiken Chemical Co. Ltd.)	• Pathogens detected by commercial LAMP kits: *Legionella*, *E. coli*, *Listeria*, *Salmonella*, and *Campylobacter* (Mori and Notomi, 2009; Niessen *et al.*, 2013)
DNA microarray	50 cfu · mL^{-1} (Rasooly and Herold, 2008)	• High sensitivity and specificity • High-throughput • Enables detection of multiple pathogens • Serotyping	• High cost of reagents and instruments • Cannot distinguish between viable or dead cells • Requires skilled personnel • Large-scale data analysis • PCR amplification may be performed prior to hybridization to increase sensitivity	< 4 hours	• Generally custom manufactured • Minimal Signature *E. coli* Array Strip (FDA-ECID) (Affymetrix) for the detection and identification of *E. coli*	• Low-medium density microarrays are preferred for foodborne pathogen testing (Rasooly and Herold, 2008)
Biosensors	Detection limit	Advantages	Limitations	Assay time	Examples of products on the market	Additional information
Optical	10^3-10^5 cfu · mL^{-1}	• High sensitivity • Real-time detection • Automated • No pre-enrichment	• High cost	45 minutes (Wei *et al.*, 2007)	Detection systems: • Spreeta (Sensata Technologies Inc.) • Biacore systems (GE Healthcare)	• *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* spp., *Campylobacter jejuni* successfully detected (Wang *et al.*, 2011; Wei *et al.*, 2007)
Electrochemical	10^3-10^7 cfu · mL^{-1}	• Real-time detection • High-throughput • Automated	• Lower specificity • Requires higher bioburden levels • Several washing steps • Sample matrix can interfere	< 1 hour (6 hours with pre-enrichment)	No commercially available unit/system	• Conductometric device successfully detected *Bacillus cereus* (Pal *et al.*, 2008) • Potentiometric sensor

		• Pre-enrichment not necessarily required	with detection			detected *E. coli* O157:H7 (Ercole *et al.*, 2003)
Mass-based	10^5-10^6 cfu · mL^{-1}	• Near real-time detection • Cost effective • No pre-enrichment	• Low sensitivity and specificity • Requires long incubation times • Many washing and drying steps	4 hours	No commercially available unit/system	• *E. coli* O157:H7 detected using a quartz crystal microbalance sensor (Fei Liu *et al.*, 2007)
Immunological	Detection limit	Advantages	Limitations	Assay time	Examples of products on the market	Additional information
Enzyme-linked immunosorbent assay (ELISA) / Enzyme-linked fluorescent assay (ELFA)	10^3-10^4 cfu · mL^{-1}	• High-throughput • Specific • Can be automated	• Lower sensitivity • Can display cross-reactivity with closely related antigens • False negative results • Pre-enrichment required • Requires skilled user	3 hours	Detection systems: • VITEK immunodiagnostic assay system (VIDAS) (bioMérieux) • Assurance Enzyme immunoassay EIA (BioControl)	• VITEK systems detect *Listeria* spp., *Campylobacter* spp., *E. coli* O157, *Staphylococcal* enterotoxins A to E and *Salmonella* spp. (bioMerieux SA, 2015c) • *E. coli* O157:H7 detection by functional nanoparticle ELISA (Shen *et al.*, 2014)
Lateral flow immunoassay	10^4-10^5 cfu · mL^{-1} (without enrichment)	• Low cost • Easy to operate • No equipment costs	• Not suitable for high-throughput screening	Assay itself takes 10 minutes, 10 hours with enrichment	• Reveal® test kits (Neogen) • VIP® GOLD™ (BioControl Systems) • DuPont™ Lateral Flow System (DuPont Qualicon)	• Commercial test kits available for *Listeria* spp., *Salmonella* spp., and *E. coli*

Growth	Detection limit	Advantages	Limitations	Assay time	Examples of products on the market	Additional information
ATP-bioluminescence	10^4-10^5 cfu · mL^{-1}	• Easy to use • Low cost • Portable devices exist • Some automated systems	• False positives from non-bacterial sources of ATP • Unreliability due to quenching or enhancement of luciferase activity or light emission • Questionable effectiveness in detecting spores (Shama and Malik, 2013)	5 minutes or up to 48 hours with membrane incubation	• 3M™ Clean-Trace™ NG Luminometer (3M) • AccuPoint® ATP Sanitation Monitoring System (Neogen) • Celsis Advance System using AMPiScreen (Celsis International) • Milliflex Rapid (EMD Millipore)	• Some units combine culture method with ATP-bioluminescence
CO_2 production	10-50 cfu · mL^{-1}	• Easy to use and minimal operator input • High-throughput • High Sensitivity	• Long assay time • Instrument space limited to 240 samples	24-72 hours	Systems: • BacT/ALERT® (bioMérieux) • BACTEC FX® (BD)	• Mainly used in sterility testing of pharmaceuticals

Table 2.9 Continued

Viability	Detection limit	Advantages	Limitations	Assay time	Examples of products on the market	Additional information
Flow cytometry	10^2 cfu · mL^{-1}	• Automation available • High sensitivity • Can detect injured/ stressed cells • Rapid measurement	• May require enrichment culture • Method for commercial kits or in-house staining requires skilled user • High cost of instrument	1-48 hours, dependent on sample type and enrichment requirement	System: • CHEMUNEX® (bioMérieux) • BD FACSMicroCount™ System (BD) • RAPID-B® (Vivione Biosciences) Kit: • LIVE/DEAD BacLight kit (Molecular Probes, Thermo Fisher Scientific)	• LIVE/DEAD BacLight kit designed for research laboratories and not suited to use on an industrial scale • Can also use species-specific fluorescent antibodies for identification or enumeration of specific species
Electrical impedance with fluorescence	10^3 cfu · mL^{-1}	• Rapid measurement • Result in real-time • Partially Automated	• Sample preparation method may require optimization • High cost of instrument	30-90 minutes	• CellFacts II System (CellFacts 2014)	• Tested in a range of industries from beverages to paint

Adapted from Law *et al*. (2015)

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) can also be used for rapid identification of bacteria. The instrument is comprised of three units: an ion source, mass analyser and detection device. A pure culture is smeared onto a target plate and exposed to the pulsed laser beam. Peptides and proteins from the cell surface are released into the mass analyser where compounds are separated according to their mass to charge ratio (Lay, 2001). The results are displayed in a spectrum and the pattern or fingerprint of peaks is compared to a database for genus and species identification. The major advantages of this method are its ease of use and rapidity. After a pure culture is obtained an identification result is available within a few minutes. However, it should be noted that spectra can differ when an organism is cultured under different growth conditions and different media (Lay, 2001; Šedo *et al.*, 2013). Although spectra will always contain "conserved" biomarker proteins, this technique is better suited to laboratories performing routine culture experiments on the same media. For instance, MALDI-TOF is widely used in clinical diagnostics but applied less in environmental microbiology where characterizing communities may require the use of a range of media types and culture conditions. Examples of MALDI-TOF MS instruments for the detection of bacteria are VITEK® MS (bioMérieux) and MALDI Biotyper (Bruker).

2.4 Conclusions and perspectives

Microbial contamination of products and processes can severely impact business operations and pose a risk to consumer health. Microorganisms can grow and survive in many environments. If not adequately controlled they can cause corrosion of manufacturing equipment and machinery, deterioration in quality of a range of products from paper and paints to fuel and baby wipes, and the spoilage of foods or personal care products leading to illnesses, outbreaks, or death. The food, pharmaceutical and cosmetics sectors are tightly regulated since they present the highest risk to consumer safety. To reduce the frequency and severity of contamination events, international organizations and governmental regulators have developed strict guidelines for business operators. HACCP is a safety and risk management plan that ensures product quality and safety throughout processing and handling and is required by law for facilities in the EU and US. Microbial analysis is used to validate plant hygiene regimes (cleanliness of surfaces or equipment), detect harmful pathogens, and is often required for product inventory release to the market. Cultivation methods have been the gold standard for

monitoring bioburden. However, the long length of time to result reduces its value and contribution to an effective HACCP plan and restricting its use to end-product testing. Both regulators and industry recognize the potential for RMMs. More rapid detection of microorganisms would allow for in-process microbial monitoring at CCPs instead of relying on control limits such as pH or temperature to assess contamination risk. Businesses would benefit from improved inventory and quarantine management. Products could be cleared for release sooner and less "safety" stock would be maintained if contamination is detected earlier and corrective actions are applied faster. RMMs could also reduce the risk of pathogen-related outbreaks from contaminated products, reduce failure costs, and brand-damaging recalls.

Despite the many advantages the uptake of RMMs for routine monitoring has been slow. A common argument from industry is concern over the regulatory status of the technology and the complicated and lengthy validation process. Pharmaceutical companies in Europe interested in implementing an RMM are required to prepare and submit a validation strategy. Some have complained about the administrative requirements for preparing these dossiers and have experienced delays of almost 18 months in receiving a reply from regulators (Miller, 2010). This experience delays validation and implementation, and could also deter the company or others in the industry from preparing new applications. In recent years regulators have been providing more guidance to both suppliers of RMMs and potential users in validating alternative methods. Some of these documents include:

- PDA Technical Report 33 - Evaluation, Validation and Implementation of New Microbiological Testing Methods
- European Pharmacopeia Ph EUR 5.1.6. - Alternative Methods for Control of Microbiological Quality
- US Pharmacopoeia <1223> - Validation of Alternative Microbiological Methods

The validation process requires that the applicant demonstrate the RMM is equivalent or superior to existing methods in practice for detection. There must also be a comparison of specificity, ruggedness (the degree of reproducibility of results obtained under a variety of test conditions) and robustness (the capacity for the test to be unaffected by small but deliberate variations in method parameters). For example, RMMs are evaluated using a spectrum of test organisms in different physiological states to mimic the heterogeneous population that is present in reality. The limit of detection is determined for growing cells and stressed or dormant cells. A major challenge for most RMMs is their increased sensitivity and recovery

compared to reference methods. Higher microbial loads may be measured that surpass existing specification and acceptance levels. This may result in the adjustment of threshold limits, which regulators recognize may be a necessity (Microbiology Consultants LLC, 2015b). An additional concern is the change in units; for instance, measurements may be in RLUs or RFUs and not CFUs. These issues can both be resolved during the validation process with correlation studies. With sufficient scientific data, the relevant regulatory body could approve a change to bioburden acceptance levels.

A further reluctance to adopt RMMs could be over the higher costs of these methods. RMMs may require a high capital cost in the first year to cover the purchase of new equipment, and there are costs associated with validation, training, and regulatory filing (Microbiology Consultants LLC, 2015a). They also typically have higher operating costs than culture methods (Pan, 2011). Quality Assurance (QA) managers must consider all aspects of the business to fully realize the potential for cost savings. RMMs are often automated and simpler to perform so there will be savings from labour costs. Cost avoidance is also achieved by increasing productivity and stock management, as discussed earlier. Another potential area for savings is in biocide management. Many industrial raw materials used in the manufacturing of products are at risk of microbial contamination and have the potential to negatively affect finished product quality. Biocides are used to control microbial growth. Viability-based methods can provide quantitative real-time information about the physiological state of the population. Cells are more susceptible to antimicrobials during active growth, which is readily detectable by flow cytometry or electrical impedance methods. These RMMs could help direct QA managers in biocide dosing. Culture-based methods require 48 hours to return a viable count. By this time a larger proportion of cells may be in the stationary phase and dosing would no longer be as effective. The next Chapter investigates the capability of an RMM to reduce failure costs and improve biocide management in the microbial monitoring of calcium carbonate slurries.

Chapter 3

Industrial case study of microbial quality control management: Omya AG

3.1 Introduction to case study: Omya AG

The following study investigates microbial quality control management strategies in industry. This discussion uses calcium carbonate slurry products produced by Omya AG as a case study model, but the observations and findings apply to the industry at large.

3.1.1 Aims and objectives

The current study aims to:

- Understand the Omya AG approach to microbial quality management
- Characterize the challenges in maintaining microbial quality in slurry
- Evaluate the effectiveness of product preservation strategies
- Reinforce the necessity for continuous and accurate product testing
- Optimize and transfer a rapid microbiological method to slurry monitoring on an industrial scale
- Evaluate the costs and quality of information from different microbial testing methods
- Identify cost savings opportunities in improved biocide management

3.1.2 The calcium carbonate market

Calcium carbonate (CC) is mined from open cast quarries and underground mines of chalk, limestone, dolomite and marble. It is used as a manufacturing and functional filler by several industries to confer desirable qualities, improve end-product performance, and replace expensive raw materials. Using paper as an example, qualities relating to whiteness, brightness, smoothness, opacity, glossiness and printability are dependent on high quality CC loading. In addition, paper manufacturers achieve considerable cost savings by replacing expensive pulp content ($680-1,010/tonne) (Schaefer *et al.*, 2013) with CC priced at $220-320/tonne USD (Industrial minerals, 2015). Furthermore, CC loading increases paper durability allowing for faster machine running speeds and drying times; this translates into decreases in energy consumption, increases in productivity and a reduction in overall production costs (Roberts, 2010; Morimanno, 2013). CC is rapidly becoming ubiquitous in manufactured products and everyday life. Other industrial applications and the properties delivered by CC are summarized in Table 3.1.

Global production of ground calcium carbonate (GCC) was estimated to be 85.7 million tonnes in 2013 (Wilson, 2015). Precipitated calcium carbonate (PCC) is synthetically produced by carbonation of hydrated lime and also used as a filler, but

has a lower production capacity at 17 million tonnes per annum (Elliott, 2012). The global market is dominated by two multinational producers, Omya AG and Imerys Minerals Ltd., but is also supported by smaller producers regionally. Omya AG is the global leader in GCC and PCC production holding 69% of the European market, 22% of the US market and 14% of the Asian market (Wilson, 2008).

The dynamics of the CC market are linked to global economic trends and fluctuations in the paper, paint, plastics and construction industries. The largest consumers by sales are listed in Table 3.1. During the recent recession carbonate sales were negatively affected by the 13 and 16% declines in paper production in Europe and North America respectively (Elliott, 2012). Although sales have recovered to pre-recession levels, in the last few years Asia became the fastest growing market and replaced Europe as the largest producer in 2013 accounting for 39% of GCC output, followed by 29% in Europe, 26% from North America and 6% from the remaining regions (Wilson, 2015). Market growth continues to be driven by increased loadings of CC in final products, the replacement of kaolin with CC in paper, and CC product innovation and improvement for targeting new applications. Economic growth in the emerging markets of Brazil, Russia, India and China (BRIC) has also significantly contributed to increased demand and consumption in the paints, plastics and construction sectors. Asia is forecasted to account for 52% of global demand by 2016 (Roskill Information Services Ltd, 2012).

3.1.3 GCC production and distribution

Calcium carbonate from Omya AG is sold as bulk dry powder and as slurries with 25-80% (w/w) solids content. Product grades differ based on particle-size distribution and/or percent solids, which deliver different properties and process-enhancing qualities to the end-users. Some business sectors, such as the paper industry, prefer dispersion products for their ease of use, pumpability and handling. This study focused on the microbial quality and microbial community of slurry sold to the paper and board industry in Europe. For paper filling applications a solids content of 60-78% (w/w) is used with 90% of the particles within the size of 1-5 μm and 60% < 2 μm. In paper coating applications the solids content is 72-78% (w/w) and the particles are more finely ground with 90% < 2 μm and 60% < 1 μm. Slurry production of all product grades follows the same general process (described in Figure 3.1). The main difference between the coarse and fine slurry products is the concentration of dispersing agents used and the duration of the final grinding step; the consequences of this will become clearer in subsequent discussions.

Table 3.1 - Industrial applications of $CaCO_3$

% $CaCO_3$ sales	End-use market (End product examples)	% $CaCO_3$ loading in final product	Functional properties conferred
39%*	Paper (Newsprint, food packaging, tissue, cigarette paper)	12-50%§	**Paper filling**: Imparts opacity, whiteness and printability; high loadings translate to faster dewatering/drying times **Paper coating**: Provides a smooth paper with superior gloss and print quality compared to uncoated paper. Good runnability on fast paper machines
26%*	Plastics and polymers (PVC pipes, food packaging films, plastic bags and bottles, nappies/diapers)	15-80%^	Delivers increased rigidity, stiffness and impact strength to final product; stabilizes rheological properties of polyvinyl chloride (PVC) plastisols; improves thermal conductivity enabling swift heating and cooling during processing (compared to polyethylene resins); provides permeability, chemical resistance and barrier properties; improves stretching and breathability in films and facilitates moisture absorption
12%*	Paints and coatings (Emulsion or automotive paints, inks)	15-60%§	Used as extenders to improve paint quality; improves opacity, matting, anti-cracking and anti-corrosive properties
23%	Adhesives and sealants (Grouts, glazing)	50-60%§	Improves rheological properties such as flow and slurry handling/pumpability; reduces water/volatile content of compound
	Construction (Concrete, asphalt, bricks)	15-80%§	Improves adhesion of ingredients in cement and concrete; increases strength, durability, rheology and workability of building materials
	Food, pharmaceuticals and personal care (Calcium supplements, chewing gum, toothpaste)	Up to 30%	Food: Food additive for calcium fortification Pharmaceuticals: Non-toxic functional filler for medical pills Personal care products: Confers whiteness, brightness and abrasive properties
	Environment (Water treatment, agriculture)	Up to 60%	Neutralizes pH of drinking water; used in desulphurization of flue gas from power stations; stabilizes pH in soil to maximize fertilizer application and efficiency

Modified from Wilson (2015). * Roskill Information Services Ltd. (2012); §Omya AG (2013); ^Feytis (2011)

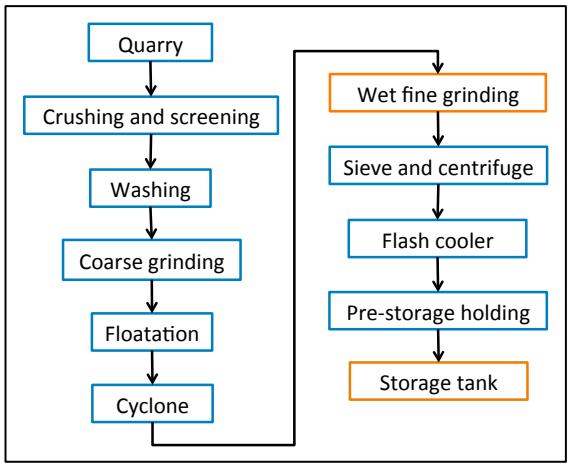


Figure 3.1 - Generalized block flow diagram of the GCC slurry production process.

Rocks are extracted from underground or open pit mines. The material undergoes further crushing and screening to remove debris and contaminants (e.g. soil) and is transported by truck or conveyor belts to the processing plant. During the washing, coarse grinding and floatation steps additional mineral impurities such as coarse clay and silicates are removed. The particulate material is separated by size in a cyclone device and the smaller particles proceed to wet milling. During the energy intensive wet fine grinding stage the temperature of the suspension is 80-110°C. Excess water is removed by sieving, centrifugation and flash cooling, where the temperature is cooled to < 70°C. The product is transferred to pre-storage tanks and dosed with preservative while the slurry temperature is 45-60°C. Final storage occurs in 1,000-3,500 m^3 tanks. Temperature is not controlled during storage and transport and is generally recorded at 25-30°C, which is optimal for microbial growth. The duration and speed of mixing is dependent on the volume of slurry in the tank and the product grade (solids content and particle fineness). Coarse products will settle faster and require more mixing. The orange boxes represent critical control points in product disinfection and inhibiting microbial growth.

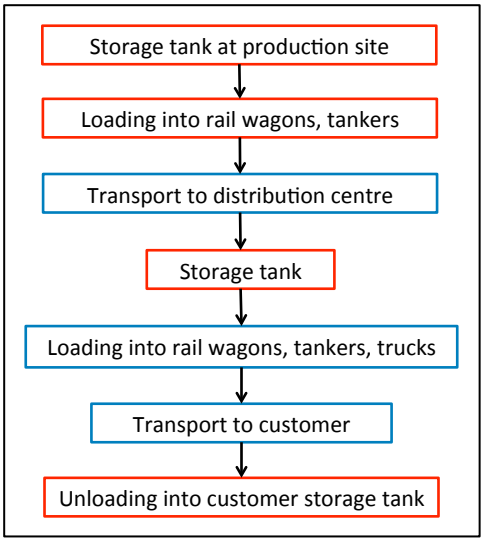


Figure 3.2 - Slurry handling and distribution from the Norwegian plant to the customer site. Freshly produced slurry is dosed with preservative and held in storage tanks. Product destined for the NLMJ distribution plant is loaded and transported by tanker for the two-day journey. Slurry is unloaded into storage tanks and held until transport and delivery to the customer site. Product quality is closely monitored. The red boxes indicate control points at which more preservative and/or adjustments to pH can be made. Omya strives for a single post-production treatment at the top-end of the lifecycle to avoid further downstream costs.

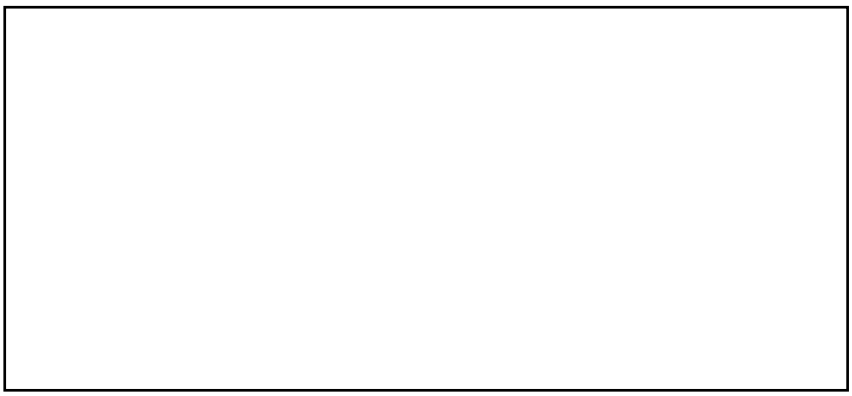


Figure 3.3 - Volume of GCC slurry manufactured by Omya in Europe (2011-2014).

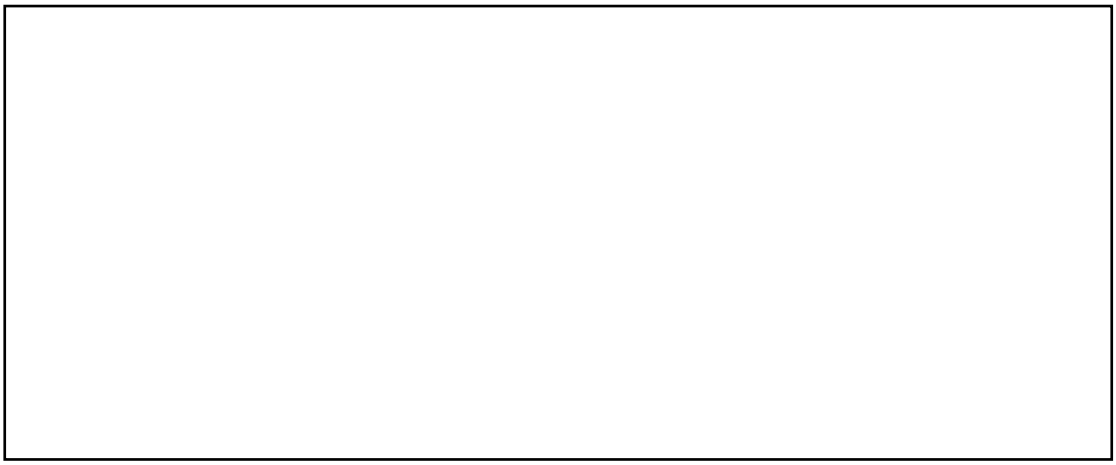
3.1.4 Consequences of microbial contamination in slurry

Slurry is used as a raw material in a wide range of manufactured products; therefore, any microbial load carried in slurry has the potential to contaminate a customer's manufacturing process and the final product itself. In spite of this, the main purpose of controlling bioburden is to maintain the physicochemical properties of slurry and ensure the functional and mechanical characteristics delivered by CC loading are achieved. Bacterial counts over 10^6 cfu mL^{-1} causes adverse changes

in pH, viscosity, colour, and odour in slurry (Schwarzentruber, 2003). Excessive microbial respiration can lead to acidification of the product. When the pH drops below 8.0, CC will precipitate out of the dispersion and viscosity can increase to the point of solidification if it is not corrected. Viscosity is also increased by polysaccharide secretion from biofilm producing bacterial species and the presence of filamentous bacteria and fungi. Generally changes in viscosity impact upon rheology and performance during handling and workability in manufacturing; however, it could result in inferior end product quality due to changes in properties such as light scattering. In extreme cases solidification of slurry could damage storage tank components and piping. Microbial contamination can also cause pigmentation of slurry and negatively affect opacity, brightness, and whiteness. High microbial loads of *Pseudomonas* sp. and *Methylobacterium* sp. can produce a pink-hue in slurry (Schwarzentruber, 2003; Di Maiuta, 2010). The fungal species *Fusarium solani* was recently isolated from slurry and also found to cause pink colourization (personal communications with J. Glaubitz, Head of Microbiology at Omya). *Pseudomonas* sp. has been associated with the production of malodourous nitrogenous compounds such as amines and ammonia. Anaerobic growth can develop during periods of stagnation in the tank, for instance when stirring has stopped or there has been no loading or unloading of product (Schwarzentruber and Gane, 2005). The growth of sulphate-reducers in such conditions can cause greying and unpleasant odours in slurries and other mineral dispersions (Salzburger, 1994). Given the undesirable effects microbial load levels can have on slurry quality, close monitoring of specifications can assure that corrective measures (discussed later) are applied in a timely manner. The failure to do so can result in customer dissatisfaction, a loss of brand reputation, rejection of the shipment, and a loss of revenue and future business. Furthermore, there is the potential for bioburden in the slurry to be carried through to finished products such as fortified foods, food packaging (Suominen *et al.*, 1997; Vaisanen *et al.*, 1991), and personal care products and pose a threat to consumer health and safety. CC destined for the food, pharmaceuticals and cosmetics industries undergoes thorough microbiological testing for detecting pathogenic organisms. In 2012 Omya alerted their customers to the presence of *Salmonella* in a shipment of CC prompting Nestlé USA to recall batches of NESQUIK Chocolate Powder over concerns of consumer safety (U.S. Food and Drug Administration, 2012). The NESQUIK product is largely marketed at

3.1.5 Sources of microbial contamination and the slurry environment

Microbial contamination occurs throughout every stage of production. The early processing steps are performed in the open environment and crushed material can be stored outdoors in heaps for several days where the surfaces are exposed to water, air and soil. Depending on the plant, the water used in washing, floatation, and grinding can be from municipal water sources or re-circulated process water. Contact surfaces or residual material on conveyor belts, process equipment, piping, and storage tanks also provide sources of contamination. Omya relies on two critical control points during slurry production for controlling microbial load (Figure 3.1). The first is the fine grinding step where the temperature of the aqueous phase may rise above 100°C. The importance of this step was revealed when process



The second control point occurs before long-term storage when preservatives are applied. Schwarzentruber (2003) described a persistent population that survived the high temperatures and identified the flash cooler as a major source of contamination. In conversations with S. Urwyler (R&D Microbiology, Omya) the flash cooler remains a significant bioburden contributor in production plants at Omya today. Despite the fortuitous disinfection provided by wet grinding, poor plant hygiene of downstream machinery and storage tanks results in further inoculation of the product. Agitation and moderate temperatures during storage and transport (25-30°C) provide favourable conditions for microbial growth. Dosage with preservatives and biocides at the end of production is vital for minimizing and controlling microbial load throughout the product's lifecycle. If required, re-preservation can also be performed during slurry loading for transport and during storage at a distribution site (Figure 3.2).

Ion chromatography analysis of slurry confirmed the presence of low levels of biologically relevant macronutrients: dissolved organic carbon, nitrogen species, sodium, potassium, magnesium, sulphate, and phosphate (personal communications with N. Di Maiuta, previously from R&D Microbiology, Omya). Schwarzentruber (2003) and Di Maiuta (2010) demonstrated that the natural organic deposits from the carbonate material, water, and chemical additives provide a suitable environment for growth of a range of microorganisms. The physicochemical characteristics of slurry are achieved and maintained by the addition of dispersing agents, thickeners and anti-settling agents. Slurries with higher solids content or smaller particle sizes contain increased levels of polyacrylic acid polymers and co-polymers, which function as both grinding and dispersing agents. These compounds help achieve particle fineness and maintain particle suspension, viscosity and rheology during extended storage. The acidic polymers are typically neutralized by sodium hydroxide or ammonia (Michl *et al.*, 2007) therefore higher concentrations of dispersants are met with increased levels of these bases. Lab managers from the European plants and North America have observed an increased tendency for contamination in finer-ground products. This could be a reflection of the increased availability of macronutrients from the higher concentration of additives used.

3.1.6 Challenges in controlling microbial growth

Despite the application of biocides and preservatives slurry quality often deteriorates over time. In an ideal scenario the preservatives dosed at the end of production would provide sufficient protection for the duration of the lifecycle described in Figure 3.2. In reality the situation is more complex with the plant managers focused on minimizing costs. Preserved slurry is still susceptible to microbial contamination during storage and transport. Each exposure to new surfaces in piping, tankers and tanks provides a potential source of inoculation, particularly if biofilm communities have been established. An added complication in controlling microbial growth in storage tanks is the changing inflow of slurry from production or delivery and outflow for shipments. Storage tanks are rarely “emptied” and continuously topped up with fresh product. Three issues must be considered when maintaining microbial quality: effective dosing of biocide, the potential for residual product to act as an inoculum for incoming slurry, and the addition of nutrients provided within the fresh product. Firstly, preservative is dosed at the minimum inhibitory concentration (MIC) necessary to inhibit growth for a given volume of slurry. When freshly preserved slurry is added to a tank containing older slurry this effective dose is diluted and

adequate protection may not be provided. The exposure to sublethal concentrations of biocide can promote biocide tolerance and resistance in the population (Russell, 2002; Maillard, 2002; Huet *et al.*, 2008). Previous work in slurry has shown that the stability of biocides deteriorates over time and populations have adapted to initially effective MICs (discussed in the next Chapter) (Schwarzentruber, 2003; Schwarzentruber and Gane, 2005; Di Maiuta *et al.*, 2009; Di Maiuta, 2010; Di Maiuta *et al.*, 2011). Therefore, it should be assumed that the biocide concentration in the remaining slurry is not optimal and calculations for dosing of the fresh product should take into account the total volume of the storage tank. Unfortunately QA managers at the plants do not always follow this recommendation. Secondly, the design of the tanks does not allow for them to be fully emptied since the piping outlet is not located at the bottom. In the case of the tank farm at NLMJ, the process of "emptying" the 1,500 m^3 tanks leaves behind 100-200 m^3 of slurry. With tankers delivering new product from Norway every few days, a continuously-fed system has been inadvertently created. Tanks are thoroughly emptied and cleaned by disinfection and physical abrasion yearly. If contamination is thought to be due to poor tank hygiene then slurry is removed and the walls sprayed with water using a high-pressure apparatus. Otherwise the residual slurry rests in the tank allowing for a microbial population to establish and/or for biofilms to develop on surfaces of the tanks and piping. This perpetuated population not only inoculates the "clean" incoming material but also thrives on the fresh nutrients introduced to the system.

3.1.7 Preservation strategies in slurry

As previously mentioned, microbial growth in slurry is controlled by use of biocides. Because of increasing regulations surrounding biocides, the alkaline environment of slurry (pH 8.5-9.5), and the requirements for thermal stability and long-term protection, the list of compatible biocides is limited. Schwarzentruber and Gane (2005) outlined the key constraints on preservatives suitable for use in mineral dispersions (refer to Table 3.2). The authors also identified a list of biocides that have been evaluated and successfully applied in slurry (Table 3.3). Blends of these compounds are preferred due to their enhanced performance and improved properties. Biocidal blends offer improved long-term and thermal stability, a combination of short- and fast-acting compounds and increased targeted sites of action in microorganisms. An additional advantage to using blends is the synergistic effect observed. When applied in combination a lower MIC of each biocide is needed to achieve the same inhibitory effect as when they are acting alone (Lambert *et al.*, 2003; Winkowski, 2004).

Table 3.2 - Criteria for biocides used in slurry

- Effective under alkaline conditions
- Thermal stability to 60°C
- Combination of short- and long-term activity
- Approval and registration with regional regulatory bodies (e.g. EU BPD, US EPA/FDA etc.)
- No adverse effects on the physicochemical properties of slurry
- No evidence of leaching or migration of the biocides into finished products (i.e. from packaging materials into foodstuffs)
- Possession of a positive redox potential
- > 80% biodegradability in closed bottle tests (according to OECD 301 guidelines)
- No inhibition of nitrification processes during wastewater treatment

Modified from Schwarzentruber & Gane (Schwarzentruber and Gane, 2005)

Omya continuously invests time and resources into selecting the biocides and blends that 1) provide the best long-term protection without compromising the physicochemical properties of slurry; 2) require the least post-production management and chemical intervention for maintaining product quality; and 3) the most cost effective solution when considered across the product's lifecycle. However, in discussions with P. Schwarzentruber, previous Head of R&D Microbiology at Omya, it was revealed that in some instances customer and industry demands can be equally influential in biocide selection. During the mid-1990s there

formaldehyde-releasers with isothiazolinones: EDDM/CMIT/MIT, trade name OmyAK (Dow Chemical Co.) herein referred to as "AK". Although these blends

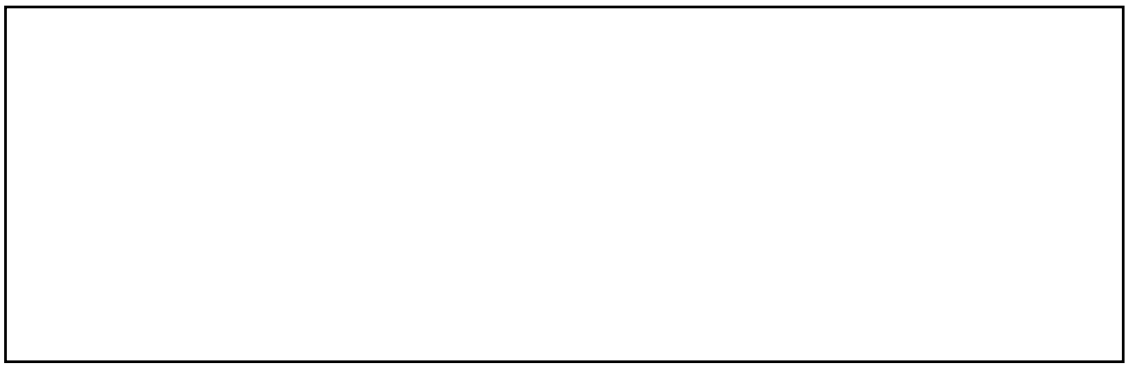
Table 3.3 - Suitable biocides for slurry preservation

Compound	Common name	Strengths	Weaknesses	Mechanism of action
2-Bromo-2-nitro-propan-1,3-diol	Bronopol; BNPD	Broad spectrum bactericide; slow-acting	Poor thermal stability; decomposition under alkaline conditions at temperatures over 60°C; poor anti-fungal activity	Electrophilic; reacts with -SH groups, disrupts protein synthesis
2,2,-dibromo-3-nitrilopropionamide	DBNPA	Broad spectrum; fast-acting; low environmental impact with degradation to CO_2	Exothermic decomposition at elevated temperatures; short-term protection; shorter half-life at pH > 8.0	Electrophilic; inhibits enzyme activity and respiration
Isothiazolones: 1,2-benzisothiazolin-3-one; 2-methyl-4-isothiazolin-3-one; 5-chloro-2-methyl-4-isothiazolin-3-one	BIT MIT CMIT	BIT - good long-term protection; MIT and CMIT - fast-acting	High potential for sensitization; not heat-stable; decomposition in alkaline conditions	Electrophilic; inhibits enzymes in respiration and protein synthesis
Phenol derivatives (E.g. o-phenylphenol)	OPP	Broad spectrum bactericide and fungicide; alkaline biocide	Low solubility; toxicity concerns; declining acceptance	Membrane-active; penetrates cell wall and inhibits enzyme activity
Aldehydes (E.g. Glutaraldehyde)	GTA	Broad spectrum; sporicidal; good activity in alkaline conditions	Toxicity concerns; sensitizing; vaporization	Electrophilic; causes irreversible cross-linking of chemical groups in proteins and nucleotides
Formaldehyde-releasers		More stable and slower acting than formaldehyde		
• Ethylenedioxy dimethanol	EDDM			
• 3,5,-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione)	DAZOMET	Optimum activity pH 4-9; good compatibility with surfactants	Decomposition is faster in alkaline conditions; intermediate breakdown products have a strong smell (hydrogen sulphide, formaldehyde, carbon disulphide)	

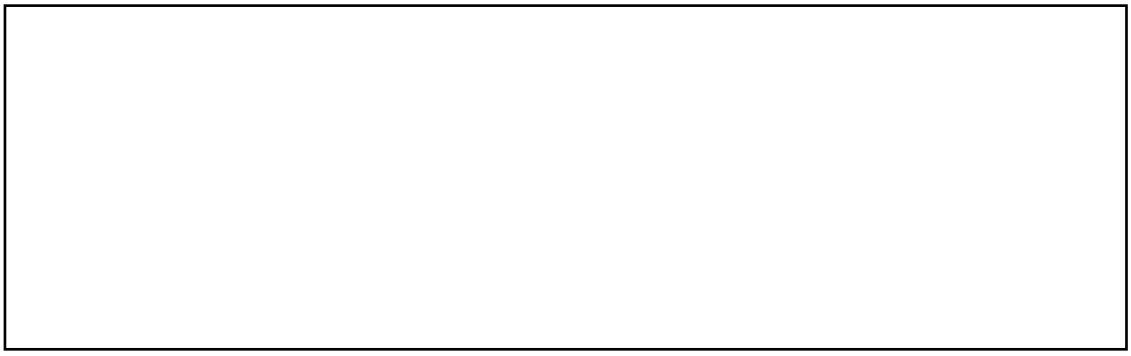
OPP has the regulatory approval for use as an industrial surface disinfectant, a post-harvest fungicide for citrus fruits and pears, for the protection of mineral dispersions, paints, and metal working fluids, and as a preservative for textiles, leather goods and wood products. Since slurry is a raw material for a number of manufactured products, it is also necessary to consider customer needs and consumer safety in regards to the finished product. Different end-use industries will face different chemical restrictions and regulations. Materials used in food packaging encompassing paper, paperboard, films and adhesives are subject to safety assessment, evaluation, registration and authorisation. The European Commission and U.S. Food and Drug Administration (FDA) take a similar stance on the safety of food contact materials. Such materials must be generally recognized as safe in food and/or for their intended use in food packaging and used in accordance with their approval; for instance, maximum residue limits (MRL) are defined for substances to establish the upper legal concentration level allowed in the final product. Furthermore, compounds must not:

1. Transfer from the packaging to the foodstuff to a concentration large enough to endanger human health
2. Cause an unacceptable change in the composition of the food
3. Negatively affect the organoleptic qualities of the food (taste, smell, etc.)
4. Have a preserving effect on the foodstuff (as a result of chemical transfer)

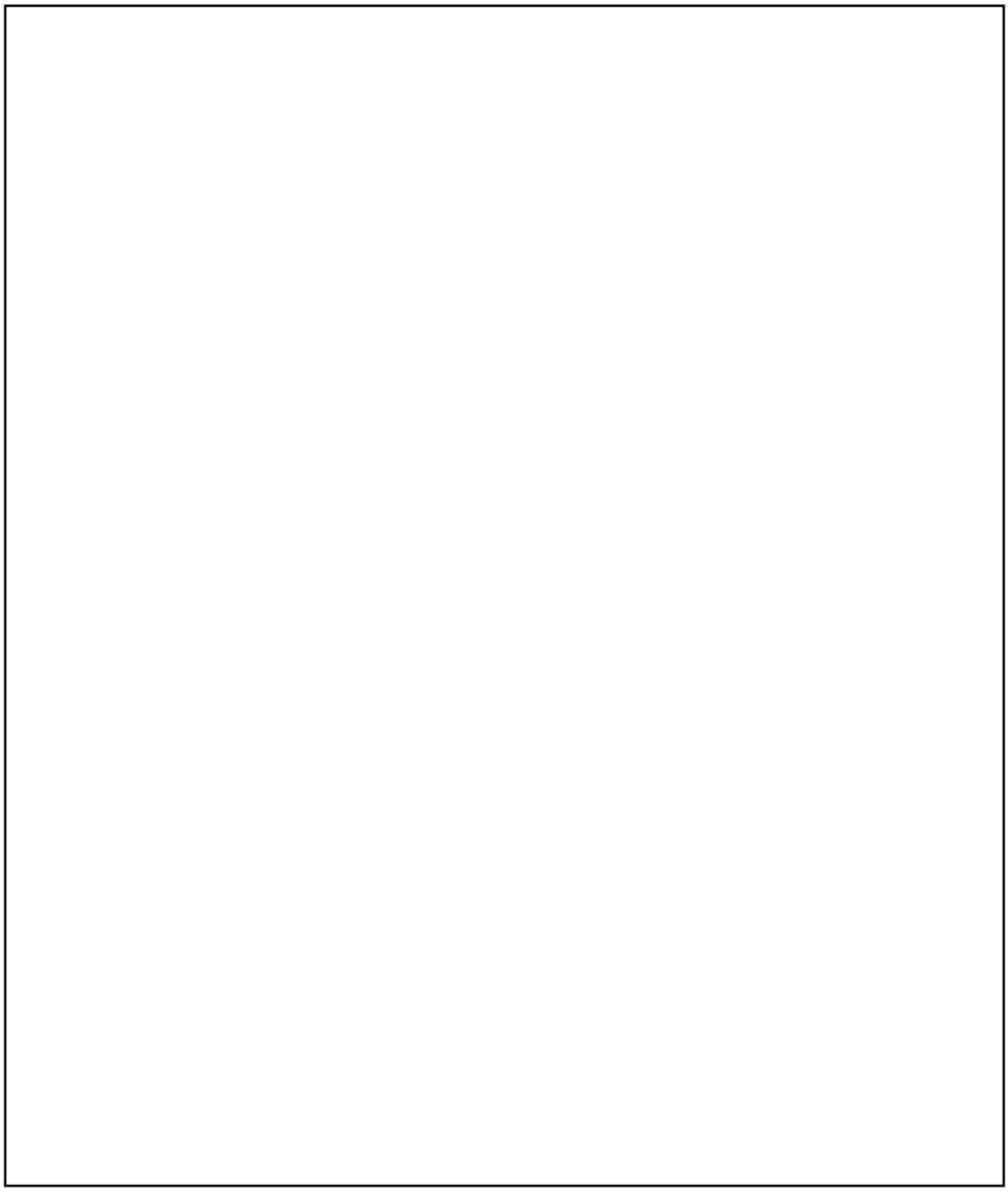
The biocide compounds listed in Table 3.3, including OPP, possess regulatory approval in the US and EU for use in food packaging. From a food safety perspective the switch to OPP was not expected to have any impact upon customers since there was no change in the health risk to consumers. In early 2007



In the course of investigating new chemical additives that offered improved dispersion properties and pH stabilization, the compound 2-amino-2-methyl-1-propanol (AMP) was identified. AMP is a co-dispersant added to paints, metal working fluids, and personal care products to stabilize emulsions at high pH, improve pH buffering, provide corrosion protection, enhance biocide activity, and reduce microbial degradation of the preserved product (Angus Chemical Company, 2015a). Most problems in maintaining slurry quality stemmed from the unstable pH. After production of AK slurry the pH is 9.0-9.5 and during its lifetime it typically declines to pH 8.5-9.0 but in extreme instances has been observed to drop to pH 8.2-8.5. It was theorized that the inclusion of AMP would help stabilize pH above 9.5, maintain slurry rheology (properties related to flow and usability) and viscosity, and eliminate the additional post-production treatments required to manage AK slurry. Preliminary tests in slurry contaminated with an AK-resistant isolate of *Pseudomonas mendocina* proved AMP enhanced the performance of AK (Di Maiuta, 2010; Di Maiuta and Schwarzentruber, 2012). Di Maiuta (2010) also demonstrated that AMP on its own has disinfection properties on this population. AMP is approved by the EU for use as a dispersion and floatation agent in the manufacture of food contact materials (BfR recommendation XXXVI). It is also approved by the US FDA as an additive in food packaging adhesives (Code of Federal Regulations Title 21 (CFR) 175.105) but is accepted as a dispersant in food contact materials under restricted conditions (CFR 176.170, CFR 176.180). Angus Chemical Company and its parent company the Dow Chemical Company clearly state that AMP is not a biocide, but is instead a “bioresistant” material “not readily chemically decomposed by microbial attack…(and) does not provide a ready food source for microorganisms” (Angus Chemical Company, 2015b). Having avoided



3.1.7.1 Expenditure on product preservation



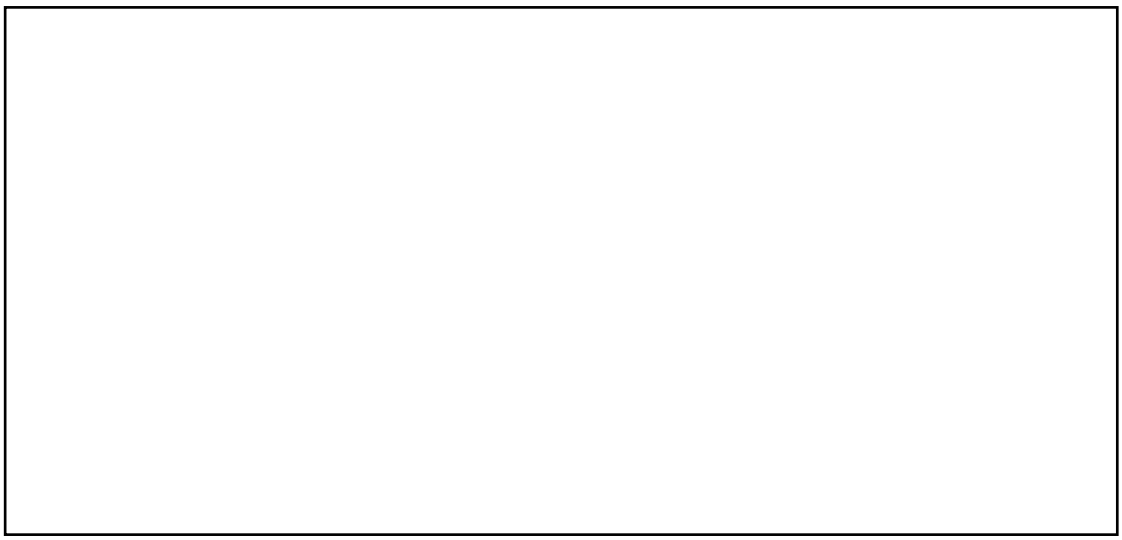
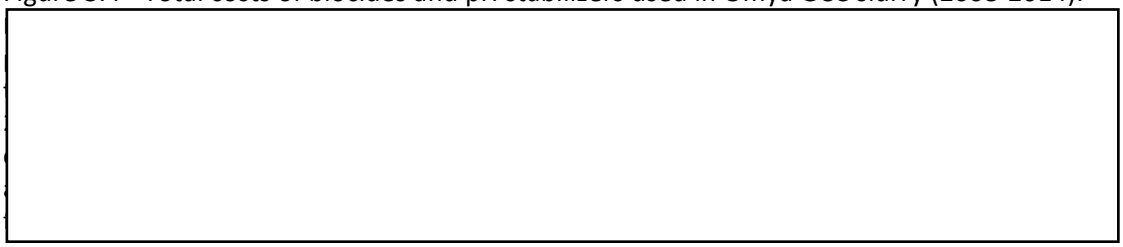


Figure 3.4 - Total costs of biocides and pH stabilizers used in Omya GCC slurry (2008-2014).



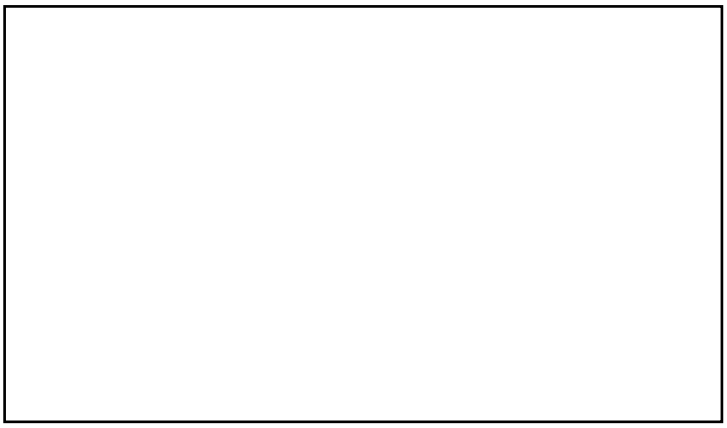


Figure 3.5 - Ratio of preservative expenses per kilogram of slurry at each plant (2008-2014).

3.1.8 Monitoring microbial contamination and the potential for rapid microbiological methods

During product storage Omya regularly monitors pH, microbial load and viscosity. When measurements fall outside of the desired specifications additional treatment and recovery measures are employed. As alluded to earlier, AK was unable to

provide long-lasting protection and slurry quality often diminished as a result of microbial growth. Bioburden is assessed using the culture-based dip slide method mentioned previously. Samples are collected daily from each storage tank and diluted slurry is exposed to the agar surface of the dip slide. Viable microorganisms present in the product form visible colonies after 48 hours of growth. Di Maiuta (2010) observed carbonate particles coated with *Pseudomonas* sp. and free planktonic cells or cell aggregates in slurry. The critical thresholds for microbial load and pH and the recommended actions are summarized in Table 3.4. When the bioburden in the product is stable and the viable cell count is below 10^4 cfu·mL^{-1} (in accordance with product specifications) QA managers interpret this is an indication that the quality control measures in place are effective. However, if excessive or rapid microbial growth is observed, the product is either not sufficiently preserved or the plant or storage tank hygiene needs attention. The most common post-treatment applied is product re-preservation with biocide. In instances where microbial growth has caused a decline in pH or discolouration, NaOH or H_2O_2 dosing may also be required (Table 3.4).

Table 3.4 - Slurry quality and microbial load monitoring and action points

Test outcome	Action
Dip slide count < 10^4 cfu·mL^{-1}	None
Dip slide count > 10^4 cfu·mL^{-1}	Monitor pH and viscosity
Dip slide count > 10^4 cfu·mL^{-1} for three consecutive days, stable pH	Dose with GTA/CMIT/MIT biocide at recommended concentration
Drop in pH over time or pH < 8.2	Correct to pH > 9.0 with NaOH
Discolouration observed	Add H_2O_2; must wait 24 hours before other corrective treatments
Dip slide count > 10^4 cfu·mL^{-1} for three consecutive days with pH drop	Correct pH first with NaOH; mix for a few hours; dose with GTA/CMIT/MIT
Dip slide counts > 10^4 cfu·mL^{-1} after biocide dosing	Repeat biocide treatment
High microbial load detected despite three consecutive doses with biocide	Send sample for analysis to R&D Microbiology; consider emptying and cleaning storage tank and pipelines

The dip slide method is the gold standard in many industries because of its low cost per test, the simplicity of the protocol, and the ease in interpreting the results. Some major disadvantages to dip slides are their underestimation of microbial load and the time delay in obtaining the data (refer to the previous Chapter for a more detailed discussion). The main challenge for QA managers at the plant is in biocide management. Biocide effectiveness will decrease as a result of tolerance and adaptation in the population, but changes in susceptibility can also be attributed to the physiological state and phase of growth of the organisms (Turner *et al.*,

2000). Exponentially growing cells are more susceptible to biocides than those in stationary phase (Russell, 2004). Once a dip slide result is available the information is two days old. A proportion of the population may no longer be physiologically active and will have lost sensitivity to the treatment. Despite this, biocide is added in order to inhibit further microbial growth and accumulation. When AK was the preferred biocide, the typical observation from the plants was a temporary reduction in dip slide counts followed by recovery of the population and the need for re-preservation. Repeated chemical intervention of storage tanks is not a cost effective solution. QA managers at NLMJ reported that controlling microbial load and pH was particularly challenging during the warmer summer months (personal communications with E. Brugman). They expressed frustration with the time delay, which made it impossible to apply corrective measures in a timely manner. Because dip slides do not provide results in real-time, decisions and actions are reactive (to old data) instead of proactive and it takes longer to uncover problems related to plant hygiene or detect the presence of a biocide-tolerant population. The delay also enhances the possibility of unknowingly shipping heavily contaminated product to a customer. For example, in 2014 a prominent paper customer encountered difficulties with a shipment of slurry. The pH was in decline and the viscosity increased to a nearly unmanageable level. Efforts made to rescue the product by adding NaOH failed due to inadequate mixing (speculation by F. Voorbraak, Laboratory Manager, NLMJ). The customer returned 1100 tonnes of slurry by barge and demanded a fresh shipment. Upon receipt at NLMJ the pH and viscosity were remedied and the product was repurposed for sale to another customer. Although bioburden measurements of the outgoing shipment to the customer were below 10^3 cfu·mL^{-1}, new dip slide counts revealed a microbial load of above 10^5 cfu·mL^{-1}. The possibility of the initial population expanding and being responsible for the deterioration of the product cannot be discounted. In an effort to stem downstream complaints of contamination, production plants may indiscriminately dose product being prepared for delivery with biocide. This may seem like a sensible solution but if the population is not sensitive to treatment then it is a waste of biocide and money.

Biocide management and maintaining slurry quality is dependent on the results from microbial monitoring methods. Dip slides are inaccurate and not sensitive enough to reliably report bioburden levels. Moreover, results are not available in real-time making it impossible for QA managers to apply biocide when it would be most effective. In contrast, viscosity measurements take a few minutes to perform and receive a result and pH is monitored in the tanks in real-time. When

values fall outside the specifications the biocide or other treatment (Table 3.4) can be applied directly and the effect can be observed shortly after. Omya could greatly benefit from adopting a rapid microbiological method for monitoring contamination. A test with improved accuracy and sensitivity and with a shorter time to result could improve biocide dosing, reduce customer complaints related to contamination, and reduce costs associated with post-production treatment.

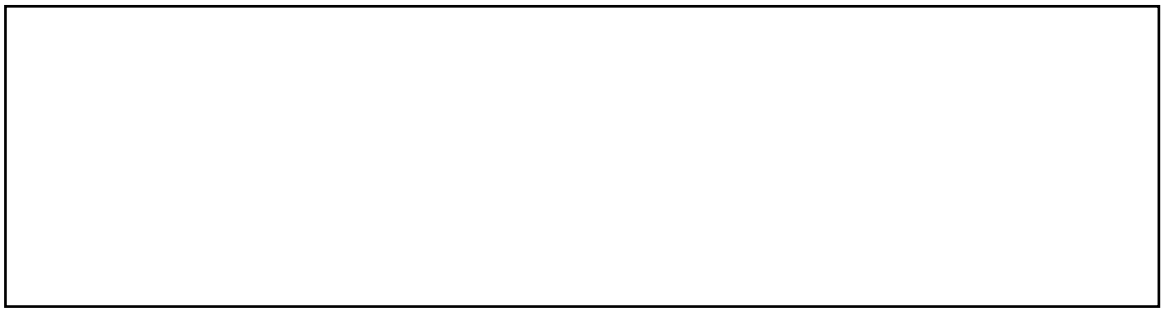
3.1.9 Strategies for ensuring customer satisfaction

Most customer complaints generally refer to poor slurry quality and shipments not meeting the agreed physicochemical specifications. When a complaint is made the sales manager responsible for that customer informs the relevant plant and logs a description of the grievance into a database. Plant and QA managers were asked to report the frequency of complaints related to microbial contamination from 2008-2014. After examining the database they revealed that no or very few complaints were attributed to high bioburden (refer to Appendix II for a summary of the responses from each plant). Instead reports of high viscosity, low pH, or bad odour, which are all indicative of heavy microbial growth, were logged as deficiencies in the physical properties of slurry. Therefore, it is likely that microbial-related product deterioration is underreported. When a more serious microbial problem is suspected such as tolerance or resistance to a preservative or heavy fungal contamination, customers are instructed to send samples to Omya's R&D Microbiology group for analysis. Laboratory results are well documented and these records may prove to be more informative in the future for gauging the frequency of microbial-related complaints. Due to logistics and geography customers can experience a considerable delay in receiving an answer or solution to a microbiological problem. They do not have time to problem solve, may not possess the know-how to rescue a non-conforming product, and cannot afford interruptions to production. Often the recommendation is to dose with biocide, however most customers feel they should not be responsible for this expense. Also some are understandably disappointed in receiving product that fails to meet specifications. In order to address these concerns the Omya's sales team developed several consignment models in order to retain customer satisfaction and loyalty. Some of the negotiable aspects relating to microbial quality management are listed in Table 3.5.

Table 3.5 - Examples of criteria regarding microbial quality in different consignment agreements
(Compiled from discussions with former and current Omya Microbiology managers)

Criteria	Option 1	Option 2	Option 3
Omya delivers product to agreed specifications (< 10^4 cfu·mL^{-1})	✓	✓	✓
Customer is responsible for the maintenance and hygiene of the slurry tanks and piping	✓	✓	✓
Product is dosed with biocide upon unloading into the customer's storage tank		✓	✓
Upon delivery of the product into the storage tank, the customer is responsible for monitoring microbial load, maintaining product stability and any costs incurred (including biocide dosing)	✓	✓	
Upon delivery, Omya is responsible for monitoring microbial load, maintaining product stability and the costs incurred until the product is used or removed from the storage tank			✓
Omya provides inspection and approval of the customer's slurry storage tank			✓

In all of the scenarios described above, Omya must deliver product with a microbial load below 10^4 cfu·mL^{-1} and customers are responsible for maintaining and cleaning the slurry storage tank at their site. The main drawback of the latter is that not all customers uphold good plant hygiene practices. If a customer's storage tank is heavily contaminated or coated with biofilms the fresh product being unloaded becomes inoculated and the population expands. It is difficult to prove the source of contamination (from Omya slurry or the customer's ill-maintained hardware). Microbial community analysis by molecular methods have been successfully employed to acquire evidence (Hubschmid, 2012) but these investigations come with some risks. On the one hand Omya could alienate customers who are not willing to admit they are at fault; on the other hand it could expose their failure to sufficiently preserve the product. If Omya maintains plant hygiene, adequately doses with preservatives, and carefully monitors the product over its lifecycle then there should be no uncertainty over product quality at delivery. To mollify some customers or those with large consignments the sales team proposed the option to have biocide dosed while the product is unloaded into the storage tank (Table 3.5, Option 2). Re-preservation is applied regardless of whether it is needed. Omya covers the expense but any subsequent chemical treatment is the responsibility of the customer. This preventative measure could have a positive impact, especially if the tank is clean and there is minimal product remaining. Realistically it would have little effect on inhibiting future growth for reasons discussed previously (such as diluting the effective biocide concentration when added to the residual product). Eliminating the treatment could save costs. For



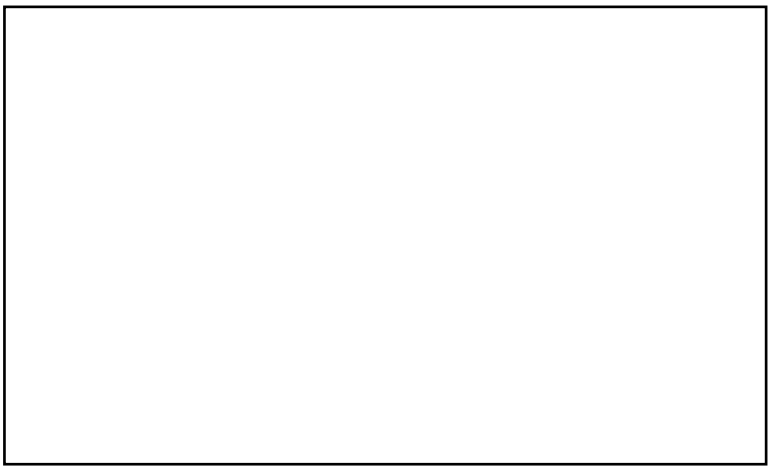


Figure 3.6 - Volume of biocide NLMJ dosed during slurry unloading to customers.

For discontented and/or select customers Omya takes over the responsibility of maintaining the stability of the product in the tank until its use (Table 3.5, Option 3). The dip slide testing, pH and viscosity monitoring is outsourced to a third-party but financed by Omya. This company is provided with guidelines and standard operating procedures for maintaining the product and applies the relevant chemical intervention as required. The difficulties in biocide management and relying on dip slides still apply but Omya is no longer in direct control of the product. They must rely on the contracted company to interpret results and apply biocide effectively. The cost of the contract can vary by customer site but the fees could total €35,000 per year per customer excluding consumables (from discussions with R&D Microbiology managers). It is difficult to identify the initial motive for devising the arrangement and since those customers are accustomed to the service it is unlikely to be withdrawn. Omya has benefited from a reduction in complaints and protected itself from accusations of contributing to a customer's microbiological problems. The agreement also allows Omya to demonstrate its commitment to delivering high quality products. Therefore, it is unclear whether the investment should be ascribed

to microbial management or to reinforcing customer relations. One could argue that there is the potential for cost savings by eliminating such contracts. If microbial quality were assured upon delivery (by adopting a more accurate and reliable detection method) then the service could become redundant. Since they are unlikely to upset current customers Omya should strive to avoid these arrangements in the future. This could be achieved by investing in rapid microbiological methods such as the CellFacts II system for monitoring contamination.

3.2 Evaluating the CellFacts II (CF) system for monitoring microbial load in slurry

3.2.1 CFII technology and Omya

The CFII system from CellFacts 2014 Ltd. measures size and counts of microbial cells in a sample matrix. It combines electrical flow impedance and fluorescent staining to ascertain microbial load. Microbial cells are isolated from the sample and stained with two fluorescent dyes. The nucleic acid stain SYTO 62 (Life Technologies) is used to detect all microbial cells within the sample, whereas the membrane-permeant cationic dye 3,3-dipropylthiadicarbocyanine iodide [diSC_3(5)] (Life Technologies) only accumulates in viable cellular membranes. The CFII software algorithm calculates TVC based on the particle count within the bacterial size range 0.72–1.50 µm and the intensity of the fluorescent signal. A defining feature of the technology is that the fluorescence corresponds to a cell's physiological state; a metabolically active cell will bind more dye particles and have a higher fluorescence level than a dormant cell (Schwarzentruber, 2003; Di Maiuta, 2010). When this fluorescence data is presented as an average for the total contaminating population it delivers insight into the activity state of the microbial load. This information could be valuable to QA managers when deciding on the necessity for and timing of biocide dosing. Another benefit of CFII is that results reflect the *in situ* state of the storage tank. Counts derived from culture-based methods only reflect the proportion of the population that was capable of growing under the defined conditions. CFII provides data in real-time and the results are available within hours instead of two days.

CFII has been successfully used to detect the microbial load in CC slurries in the laboratory (Schwarzentruber, 2003; Schwarzentruber and Gane, 2005; Di Maiuta, 2010). CellFacts 2014 Ltd. carried out short-term evaluation trials at several production sites, but NLMJ was the first to commit to incorporating the method into its daily quality-monitoring regime. The technology was used from 2009-2013 and

faced a number of challenges during its transfer into industry. During this case study significant improvements were made, namely optimization of the sample preparation and handling method to facilitate a decreased cost per test (Table 3.6).

Table 3.6 - Key milestones of the pilot study

- Worked with CF to improve and optimize the sample preparation method
- Evaluated the applicability, ease of use, and cost per tests for the two CF methods
- Decreased the cost per test from €11 to €4
- Oversaw the transfer of the new method to the plant
- Worked with CF to improve data interpretation and identify action points for QA managers
- Revealed the inadequacies of dip slides
- Demonstrated that CFII can be used as a daily monitoring tool on an industrial scale

3.2.2 Introduction to NLMJ pilot study

NLMJ is a tank farm site that facilitates the storage and distribution of over 900,000 tonnes of slurry annually to over 40 customers across Western Europe. Dip slide tests and monitoring are performed daily for 13-16 storage tanks. QA managers at NLMJ appreciate that dip slides are easy to use, take little operator time, are straightforward to interpret, and have a low cost per test at €2. However, queries from some customers revealed discrepancies between NLMJ dip slide counts and customers' TVC counts from other methods such as 3M™ PetriFilm™ count plates. The bioburden was thought to be within the acceptable limit leaving the site, but upon arrival at the customer it was claimed to be over 10^4 cfu·mL^{-1}. A likely explanation for the conflicting data is the differences in sensitivity of the tests.

NLMJ was interested in testing CFII with the intention to replace dip slides. NOME and ATGU had several criticisms of the technology after their brief experiences with it. Their feedback listed the complex sample preparation procedure and considerable operator skill and time involved to be major issues. NOME used CFII to assess microbial load before slurry shipment. They acknowledged its usefulness in instructing biocide dosing and saw there was the potential to save costs by eliminating unnecessary re-preservation. Ultimately, the CFII method as it was then was not suited to a large number of samples (over 50 tanks). NLMJ managed better in preparing the samples since it had fewer tanks to monitor. By adopting a more sensitive method than their customers they hoped to become more confident about the quality of outgoing products and reduce the frequency of customer complaints. Still, for CFII to be considered as a viable alternative to dip slides and for it to become more attractive to other Omya sites the cost per test needed to be decreased and the sample preparation method simplified.

Between the NOME and NLMJ trials the instrumentation was upgraded to include an automated fluid-handling system. This helped reduce operator input and was received positively by NLMJ.

A three-week pilot study was carried out with the following objectives:

1. To evaluate the use of CFII in general as a tool for real-time analysis of the microbial state of the product
2. To assess the potential of a new sedimentation-based sample preparation method

Dip slides, culture plate methods and the "old" and "new" CellFacts procedures were carried out on 13-15 products daily and were evaluated on the basis of:

1. Level of expertise necessary to prepare samples and run instrument
2. Operator time and commitment required to perform procedures
3. Ease of interpretation and inter-comparison of data from the different methods
4. Cost per test (inclusive of consumables, operator time, shipping, and service contract)
5. Scalability

3.2.3 Overview of the two CFII sample preparation methods

For most of the slurry products the distribution of the particles falls within the 0.50-2.50 µm size range. The majority of bacteria are 0.74-4.00 µm (Maillard, 2002) and previous work in slurry showed that most detected organisms are within 0.74-1.50 µm. Since the number of carbonate particles far exceeds the number of microbial cells, microbial cells must be separated from the slurry matrix before analysis on the instrument. The "old" sample preparation method, herein referred to as the "Histodenz" method, included a cell extraction step using the non-ionic density medium Histodenz (Sigma-Aldrich). This technique has been widely used to separate bacterial cells from soil particles (Lindahl and Bakken, 1995; Courtois *et al.*, 2001; Williamson *et al.*, 2011; Robe *et al.*, 2003). A diluted slurry suspension was deposited onto a layer of Histodenz in a tube and centrifuged. Particles were separated according to their buoyant density: bacterial cells were recovered from the supernatant above the Histodenz cushion and carbonate particles settled to the bottom of the tube. The detailed protocol can be found in the Materials and Methods Chapter and an overview of the process is shown in Figure 3.7. The main criticisms of the method were its complexity, the many pipetting steps involved, and the length of time required to carry out the procedure. There were certain steps that were highly sensitive to an operator's technique and could lead to a discrepancy in the data between two individuals preparing the same samples. For instance, inaccurate pipetting of the cell-containing Histodenz phase layer or the addition of inconsistent volumes of fluorescent dye to each sample had a significant influence

on the final result. A new method was required that reduced operator involvement and was easier for unskilled individuals to perform. This protocol eliminated the Histodenz step and relied on sedimentation by gravity for separating the larger carbonate particles from the microbial cells. After preparing the dilutions, positioning the required tubes within the racks on the workstation, and programing the software, the user leaves the instrument to carry out the rest of the sample preparation steps (Figure 3.7). Upon completion of the measurements and analyses the software publishes a results spreadsheet containing the total particle count, TVC, and average fluorescence for each sample.

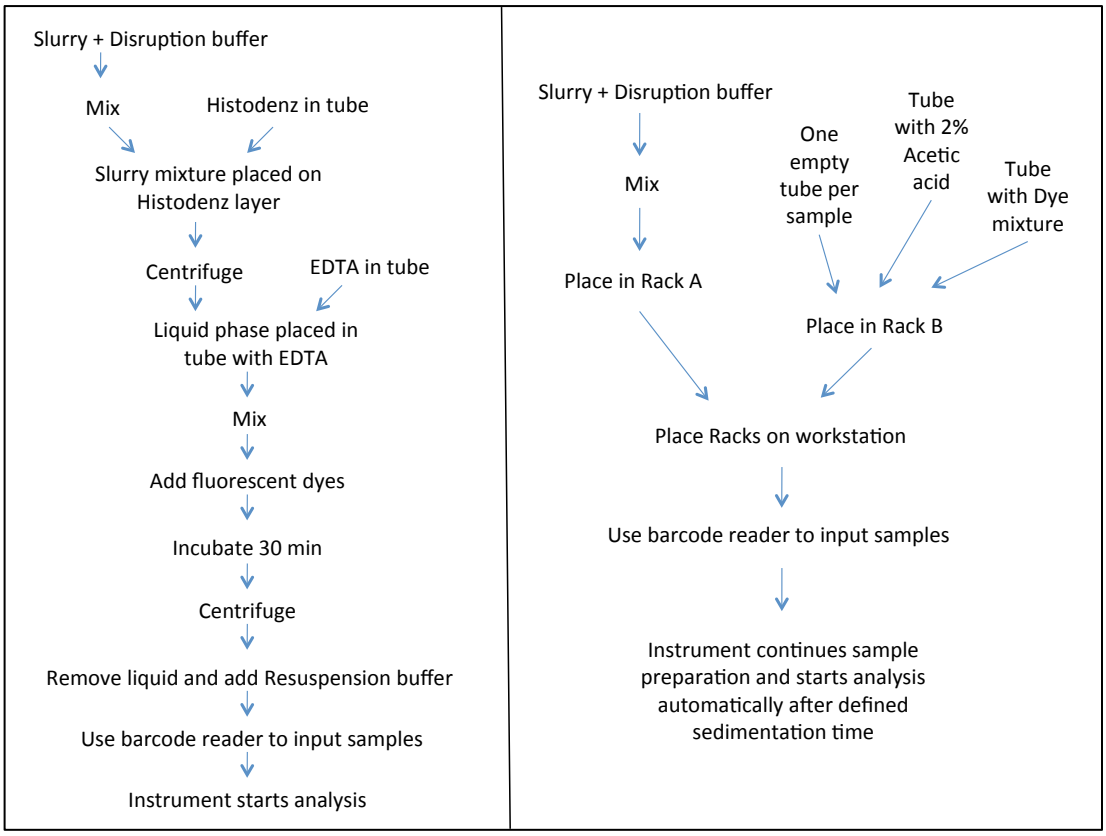


Figure 3.7 - Flowcharts for the CFII sample preparation methods.
The Histodenz method (left) requires considerable operator time. The preparation of 13-15 samples takes 3 hours. The method is prone to individual-error, laborious, and lengthy. The Sedimentation protocol (right) uses less operator time. Preparing 13-15 samples takes 30 minutes. Once the instrument has been successfully programmed the operator is able to attend to other tasks while the instrument carries out the remainder of the sample preparation.

3.2.3.1 Interpreting CFII results

To simplify data interpretation, CF implemented a colour-coding system to quickly inform the QA operator on the bioburden state of each sample. The colours are based on the calculated TVC and the threshold limits were set according to the $<10^4$ cfu·mL^{-1} specification for microbial load (Table 3.7). The CFII algorithm filters the raw data to count only those particles within the bacterial size range that possess a high fluorescence. Fluorescence intensity indicates the degree of dye uptake. Physiologically active cells possess a higher membrane potential than resting cells and should accumulate more dye. Hence, a high average fluorescence measurement should signify the presence of an actively growing microbial population and a lower fluorescence represents a less active population. This distinction could help quality managers determine the optimal timing of biocide addition. Another strength of CFII data is the ability to observe trends and problems as they develop. This is best achieved by looking at the raw data.

Table 3.7 - CF colour coded thresholds and recommended actions

Viable count per mL	Average Fluorescence	Remarks	Recommended action
< 5.9E+03	<89	Product is successfully preserved	None
6.0E3 to 8.9E+03	90 to 109	Could indicate microbial growth	Monitor pH, viscosity and subsequent TVC measurements
> 9.0E+03	> 110	Product at risk and perhaps not sufficiently preserved. Microbial load could exceed 10^4 cells/mL	After three consecutive "red" results dose with GTA/CMIT/MIT biocide at recommended concentration

The raw data is visualized with the "CFII View" software in the form of graphs plotting the count per milliliter versus particle-size or versus fluorescence channels. These plots give more specific insight into the physiological state of the population. Most bacteria replicate by binary fission where the cell elongates during growth prior to cell division. This is observable in CFII View particle size distribution plots where the distribution peak shifts to the right as cells elongate. After separation of the daughter cells and when the population enters the stationary phase, the peak position of the distribution shifts back to the left because of the smaller size exhibited by a resting cell. By looking at the raw data profiles it is possible to visualize subtle changes in the population that may not be as evident from the TVC count alone. From a microbiological perspective the Histodenz and sedimentation methods yielded comparable results. The calculated TVC and size distribution profiles exhibited the same trends. Whereas dip slides were neither sensitive

enough to provide an accurate TVC nor able depict to population dynamics within a sample.

3.2.4 A comparative assessment of dip slides, culture plates and CFII

During the study, samples were collected from 13 storage tanks daily and prepared for culturing on dip slides, plates (plate count agar, PCA; and tryptic soy agar, TSA) and for analysis on CFII (refer to the Materials and Methods for detailed protocols). From the 13 tanks nine different slurry products were tested. These differed slightly in solids content, particle fineness, and preservative method (AK-preserved or AMP). There were some products that never or only occasionally showed minimal growth on dip slides and slight growth on plate culture media (< 10^2 cfu·mL^{-1}). The corresponding CFII data detected counts between 7.0+E02 and 8.5+E03 cells·mL^{-1}, which is a more realistic and accurate assessment. Slurry is not manufactured or stored in sterile conditions; therefore it is expected to contain a microbial population. Dip slides are misleading and the concept of "no visible growth" has been engrained in QA managers to indicate the absence of microorganisms in a sample. Although culture plates may provide better nutrient conditions than dip slides, TVC estimation from plates is still inaccurate and underestimates bioburden levels. Some slurry products had highly variable and inconsistent counts from dip slides and plates while CFII measurements were more stable.

Figure 3.8 displays measurements taken from one tank over a 16-day period. In the first seven days the culture data indicated the presence of an expanding population. The time delay from incubating the dip slides meant that TVCs from Day 7 were only available on Day 9, at which point QA managers responded to the high counts by dosing the tank with Rocima 610 (R610) (The Dow Chemical Company), which is a blend of GTA/CMIT/MIT (Table 6.2). Subsequent dip slide and plate counts revealed that the population was sufficiently damaged to impair recovery and regrowth in culture (Days 10-16, Figure 3.8). The particle size distribution profiles on CFII enabled visualization of the population and minimal variation was observed from day-to-day (Figure 3.8). When a population is actively growing, the CFII profile shapes changes with cells becoming elongated and the total number of particles (representing the area under the curve) increasing in size. An example of this is presented in Figure 3.9. Since the profiles in Figure 3.8 overlay each other and the TVCs calculated by the CFII algorithm remained around 10^4 cfu·mL^{-1}, the interpretation was that the population was stable. When R610 was added there was no change to the distribution of particles; for instance, if cell lysis

had occurred this would have been reflected as a general decrease and shift in cell size accompanied by the gradual loss of cells in the following days' measurements. Indeed, the TVC did not decrease after biocide addition. Glutaraldehyde is the main component of R610 and, as discussed later in Section 4.2.2, the cross-linking effect would have prevented cell lysis. Although it would have been expected for the fluorescence and TVC to drop (since dead cells lose membrane potential and should accumulate less dye) it is possible that the biocide permeabilized and fixed the membrane, which facilitated continued binding of the fluorescent probe. This is also discussed in more detail in Chapter 4.

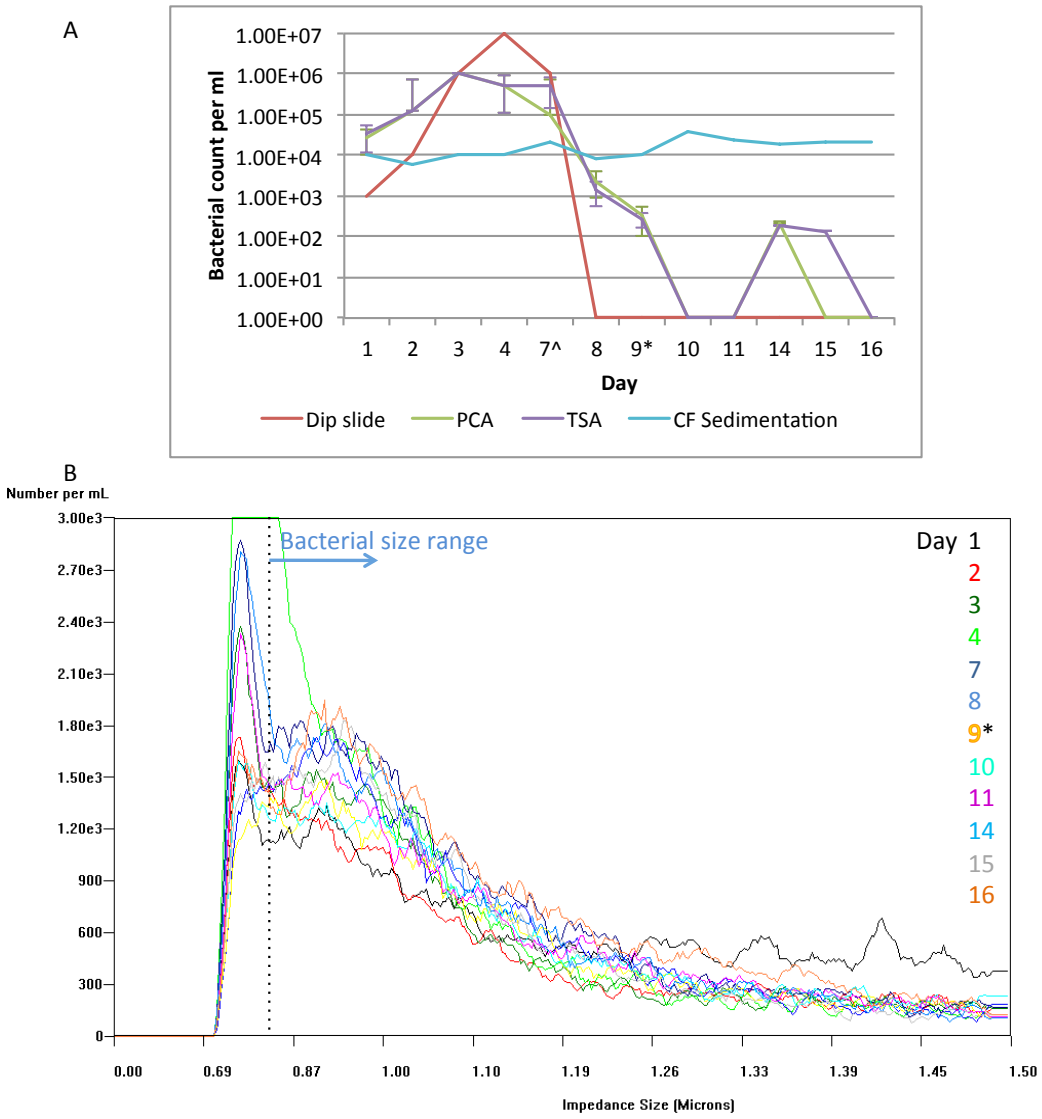


Figure 3.8 - CFII provided a more realistic estimation of microbial load.

A) Dip slides and culture plates (PCA and TSA) indicated a high bioburden with a 2-log increase in the counts between Days 2 and 3 which inexplicably dropped from 10^6 cfu·mL^{-1} to 0 between Days 7 and 8. The biocide Rocima 610 was added on Day 9 denoted by (*) in response to high dip slide counts of the previous week and when the result from Day 7 became available. The CF TVC was stable around 10^4 cells·mL^{-1}. Error bars for the plate counts are shown, but replicates were not performed for dip slides or CFII measurements. B) CFII particle distribution profiles for the same sample. The daily profiles overlay each other further suggesting a stable population. Biocide addition appears to have had no impact on the CF TVC or distribution of the particle profiles. This could be due to the fixation of cells by glutaraldehyde, which would account for the absence of cell lysis and a profile shift indicating decreasing cell size.

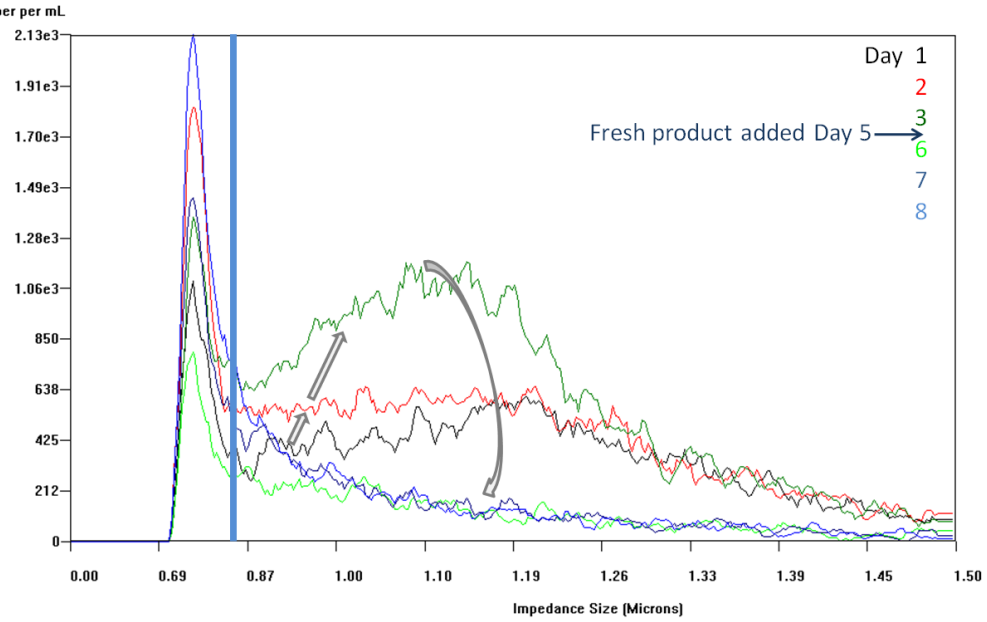
3.2.4.1 Monitoring trends in the contaminating population

CFII is able to detect subtle changes in population dynamics that are not perceptible from analyzing dip slide counts. For instance, by plotting and analyzing CFII profiles it is possible to see a population expand and then become diluted when fresh product is added to the tank (Figure 3.9). This trend is not reflected in the dip slide counts; only CFII revealed the state of the population in real-time. QA managers could make use of this capability for identifying the requirement of and timing for biocide addition. After receiving the Day 3 result, and having known that new slurry was arriving, they should have dosed the tank with biocide. Instead, fresh slurry brought fresh nutrients that would have allowed the population to continue growing (Figure 3.9). Even if the incoming product was adequately preserved, its effective biocide concentration would have been diluted and insufficient to inhibit growth or kill cells.

In another example, CFII and dip slides were used to check the quality of slurry coming from the NOME plant. QA managers received complaints from a customer of a high microbial load in a particular slurry product. Dip slide counts at NLMJ were occasionally above 10^4 cfu·mL^{-1} but not consistent enough to prompt re-preservation with biocide. The fluctuations could have been due to the high volume going in and out of the tanks every few days. NLMJ decided to check the quality of the incoming slurry before it was loaded into the tank. They wanted to know whether the observed contamination was inherent in the product already or if the problem originated within their own tanks. Samples were analysed for four days before the tanker arrived. Due to the popularity of the product the stock was stored in two tanks. The dip slide counts were low in both tanks but the duplicate samples collected from the ship reported a count of 10^4 cfu·mL^{-1} (Figure 3.10). These data suggested that the fresh product was more contaminated than the stored product, however the CFII counts indicated the opposite was true. The TVC and particle size profiles both showed that the microbial load in the tanks was higher than the product coming from the tanker vessel (Figure 3.10). In addition, the average fluorescence values are higher for the slurry in storage. Since this correlates with physiological activity it could also be stated that the microbial population was more active in the storage tanks. This information is invaluable to managers because it holds them accountable for maintaining product quality. For these two tanks they were instructed to consider treating with biocide or to clean the tanks in the near future. It should be noted that CFII analysis of other slurry products and fresh shipments showed that the incoming material was slightly more contaminated. With this evidence it was possible to report back to NOME and request better cleaning of the

tanker and/or biocide dosing of specific products prior to shipment. The ideal scenario would have been if NOME also used CFII. This could have better ensured hygiene maintenance and product quality upstream and through the rest of the product's lifecycle.

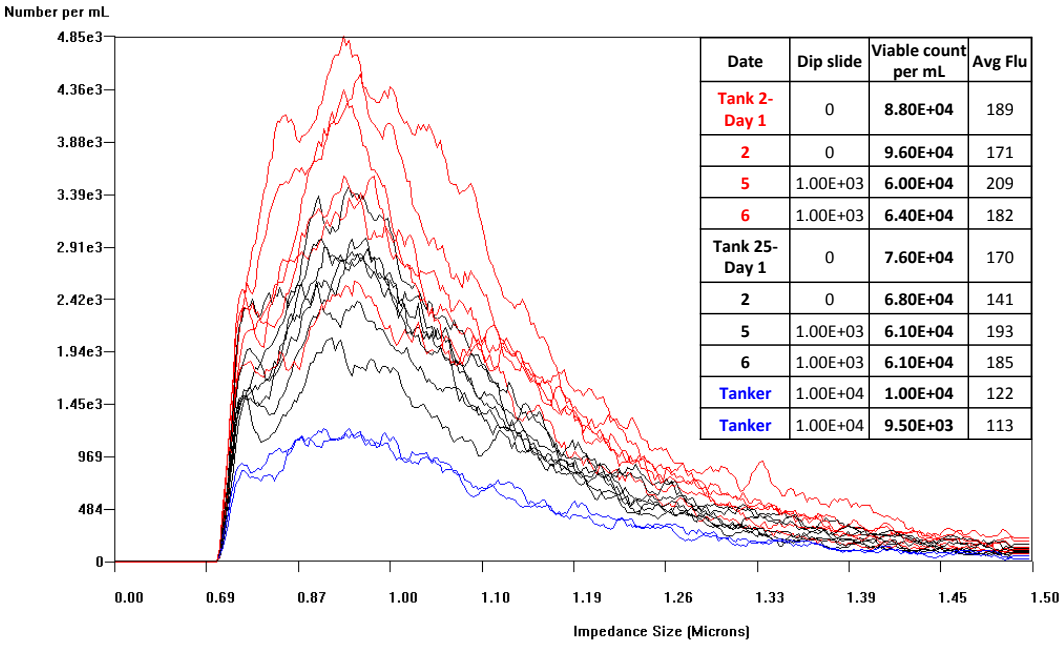
A



B Viable count per ml

Day	Dip slide	CFII
1	0	6.40E+03
2	1.00E+03	8.50E+03
3	1.00E+03	1.10E+04
6	1.00E+04	4.60E+03
7	1.00E+03	5.10E+03
8	0	5.10E+03

Figure 3.9 - Impact of fresh product addition on cell count
A) CFII size distribution profiles from Days 1-8. The microbial population increased in number and the visible trend in increasing impedance size suggested these cells were in a phase of growth Days 1-3. Fresh product was added on Day 5, and subsequent measurements on Days 6 through 8 showed the diluted population. The grey arrows help illustrate this trend. The blue line denotes the lower end of the bacterial size range. B) Recorded cell counts from dip slides and CFII from Days 1-8. The CFII counts accurately reflected what was observed in the profiles in (A). Dip slides did not capture these changes to the population.



Date	Dip slide	Viable count per mL	Avg Flu
Tank 2-Day 1	0	**8.80E+04**	189
2	0	**9.60E+04**	171
5	1.00E+03	**6.00E+04**	209
6	1.00E+03	**6.40E+04**	182
Tank 25-Day 1	0	**7.60E+04**	170
2	0	**6.80E+04**	141
5	1.00E+03	**6.10E+04**	193
6	1.00E+03	**6.10E+04**	185
Tanker	1.00E+04	**1.00E+04**	122
Tanker	1.00E+04	**9.50E+03**	113

Figure 3.10 - CFII data shows that stored slurry has a higher microbial load.
Although the dip slide counts in the table suggested the incoming product from the tanker contained a higher bioburden, the CFII cell counts and particle profiles showed the reverse. The incoming slurry was less contaminated than the product in the storage tanks.

3.2.5 Evaluating preservation strategy effectiveness using CFII

Laboratory and QA managers at several sites remarked that finely ground products tended to be more difficult to control than coarser products. These observations were made from dip slide data and years of experience. This trend was also seen in CFII raw data profiles and fluorescence plots, although the proof was made more evident by the increased sensitivity of the technology. The data from four AMP products and one AK-preserved slurry was plotted over 50 days (Figure 3.11). The plots provided a visual representation of the varying degrees of contamination and physiological activity in five product types. Of the pH stabilized products, the finer-ground HC90 and CC75 supported the greatest baseline bioburden and also had the highest numbers of highly fluorescent cells. The HC95 AK product is also finely ground but was less contaminated and the population was less physiologically active (as indicated by the lower cell numbers exhibiting strong fluorescence). HC60 and HC75 products were the coarsest products tested and had the lowest microbial load. The fluorescence profiles suggested that HC60 was more stable and adequately preserved due to the tighter and lower range of cells detected with fluorescence.

Another interesting observation from the pilot study was that some slurry products of the same grade but different methods of preservation possessed

different levels of contamination. Besides the preservatives AK and AMP the precise variation in chemical composition of the products is unknown to the author. However, J. Glaubitz disclosed that the excellent co-dispersion properties of AMP had facilitated the reduction and/or elimination of other dispersion aids in the formulation. Bioburden levels are dependent on the availability of nutrients, some of which have been attributed to the chemical additives. It must also be restated that AMP is not marketed as a biocide, therefore a higher microbial load in these products could also be the result of an absence of antimicrobial activity. Indeed, some AMP versions of a product were revealed to carry a higher microbial load than AK while others were equally preserved. Managers considered coarse products like HC60 to be more stable, requiring less post-production treatment than other grades. CFII measurements showed that pH stabilized coarse products were similarly stable and sustained a low bioburden. The particle size profiles and the detected fluorescence pulses from two tanks of AMP and AK HC60 products were plotted on the same graph over a 50-day period (Figure 3.12A). The profile shapes and distributions overlaid each other indicating that the particle counts and measured cell sizes were similar. A yellow hashed line was drawn on the graph to highlight this trend in the tanks. In contrast, the fine-ground HC90 products preserved with AMP supported a higher microbial load and higher level of fluorescent pulses compared to those treated with AK (Figure 3.12B). The areas under the curves for the time period were greater for the AMP samples. The particle counts were higher and greater dye accumulation and fluorescence was detected in AMP. The solid yellow line in Figure 3.12B represents the trend observed in the AMP tank and the hashed line shows that of the AK tank.

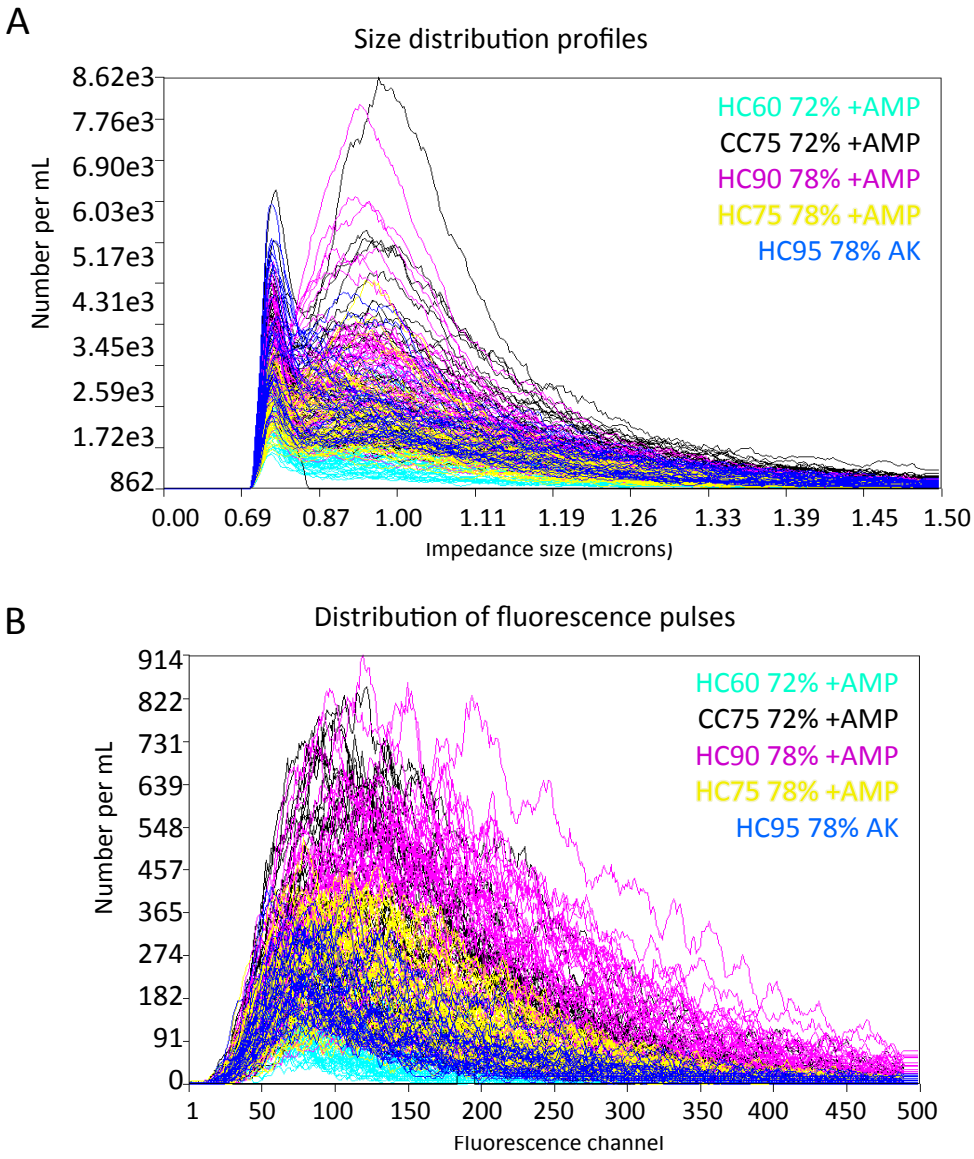


Figure 3.11 - CFII particle size and fluorescence distribution plots for different slurry products.

A) The size distribution plots showed that HC60 had the lowest baseline microbial load, followed by HC75 and HC95. CC75 and HC90 displayed greater variation in bioburden from day to day, but on average supported a higher load compared to the other products.

B) The fluorescence plots correlate with the size plots illustrating that HC60 had bioburden with low cellular activity (denoted by less dye accumulation). HC75 and HC95 possessed slightly higher levels of activity, while the contaminating cells in CC75 and HC90 showed the most dynamic range in fluorescence and were the most active and highly fluorescent.

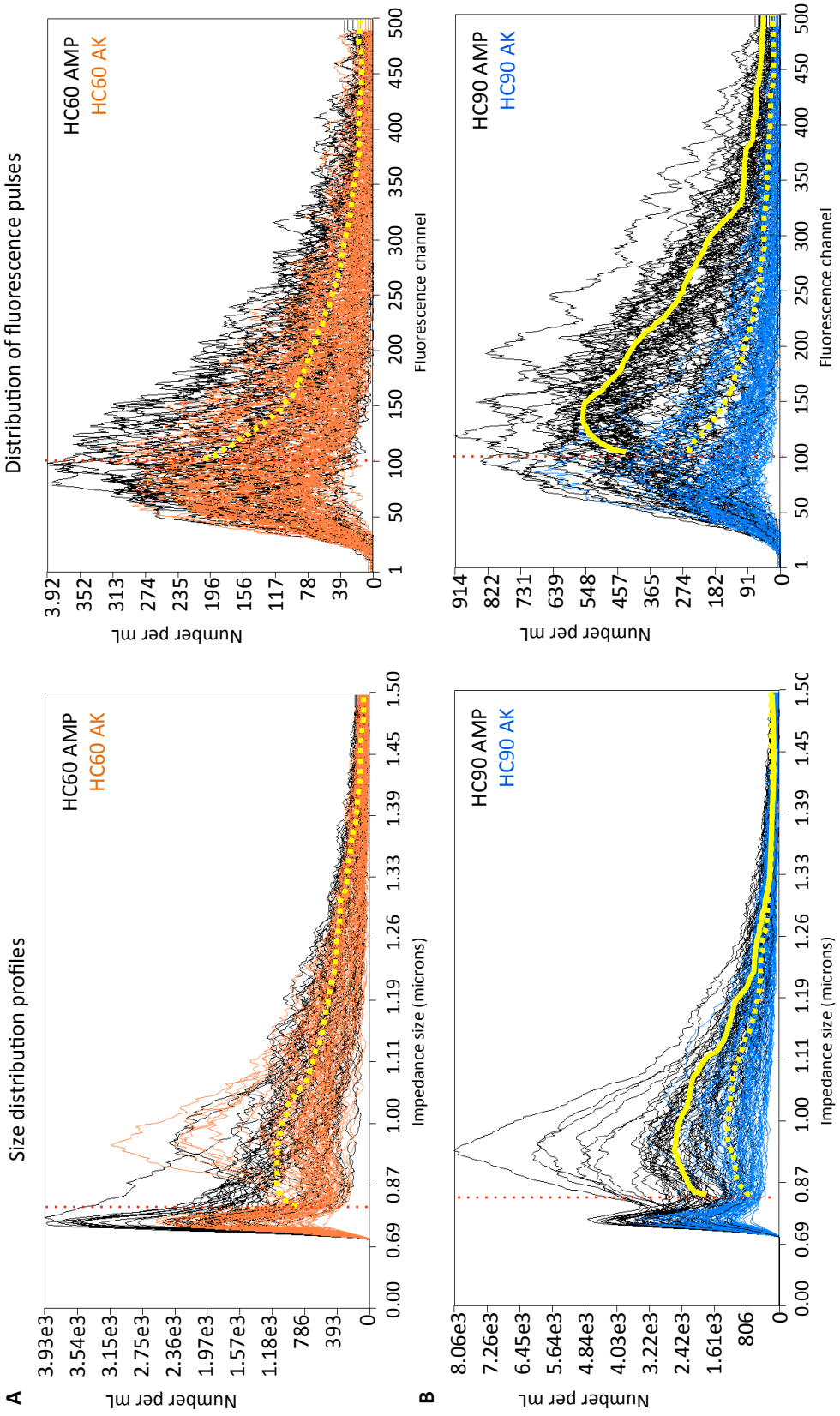


Figure 3.12 - Comparison of bioburden and cellular activity in AK and AMP products.
A) HC60 slurries were equally preserved by biocide and pH stabilizer. The number of cells exhibiting high fluorescence was comparable, which indicated the populations were stable and equally active. The dotted yellow line represents the similar average profile distributions of HC60 AMP and AK products over the given time period. B) HC90 AMP products supported a slightly higher bioburden

with a higher number of cells displaying strong fluorescence. This indicated that while the total cell count may have been similar between the AMP and AK products, the cells in AMP were considered to be more physiologically active. The solid and dotted yellow lines denote the rough averages of HC90 AMP and AK, respectively, during the examination period. The area to the right of the red vertical line in the size distribution plots and fluorescence plots corresponds to the bacterial population and significant cellular fluorescence respectively.

The main benefit of AMP is in its ability to stabilize the pH and maintain the rheological properties of slurry. When AK was the principal preservative, even if dip slide counts were low microbial contamination was evident from deteriorations in viscosity and pH. The enhanced stability from AMP masks true bioburden levels. NLMJ had received complaints from a few customers about high contamination in the fine ground product HC90 AMP, but dip slides rarely showed high counts. As demonstrated in Figures 3.11 and 3.12 CFII indicated that HC90 AMP contains a higher microbial load than other products. Feedback from one customer revealed they had cultured slurry without diluting it. The protocol for dip slides is to dilute 1:10 prior to slide preparation. This step is particularly helpful for slurries because it reduces the viscosity and opacity and improves colony visualization. When NLMJ cultured undiluted slurry they observed cases of 2- to 4-log increases in TVC compared to counts diluted in 0.9% NaCl (Table 3.8). The most likely explanation for this is that cells are damaged during dilution and exposure to dip slide media at neutral pH. AMP products have an innately higher pH than AK. While AMP is often stabilized at pH 9.5-10.0, AK pH must be maintained by NaOH treatments and fluctuates between pH 8.5-9.5. Microbial cells in AMP will experience a significant pH shock during dip slide preparation and may not be able to recover on the pH neutral media. In contrast, when undiluted slurry is cultured the carbonate provides a buffering effect reducing the pH shock and providing more favourable conditions for growth. Some AK slurries were also found to suffer from this effect (from discussions with F. Voorbraak).

Table 3.8 - Dip slide counts per mL for undiluted and diluted HC90 AMP (May 3-16, 2012) (From communications with E. Brugman)

Date	Undiluted	Diluted
03-May	1.00E+03	0
04-May	1.00E+03	0
07-May	1.00E+04	0
08-May	1.00E+04	1.00E+03
09-May	1.00E+04	0
10-May	1.00E+04	0
11-May	1.00E+04	0
14-May	1.00E+05	1.00E+03
15-May	1.00E+06	1.00E+04
16-May	1.00E+06	1.00E+04

At NLMJ AMP continues to be recognized as an effective dispersion stabilizer but its efficacy in inhibiting and controlling microbial growth is questioned. With the CFII data and the awareness of a pH shock effect, QA managers have modified their dip slide preparation practices. To achieve more accurate TVC estimates all AMP products with the exception of the finely ground HC90 are cultured undiluted on dip slides. HC90 AMP is diluted in order to restrict the frequency of high bioburden observations. The justification for this is that managers needed a more clearly defined action point for biocide addition in this product. Since undiluted HC90 regularly registers TVCs over 10^4 cfu·mL^{-1} it is difficult to identify the timing for re-preservation. So long as the physiochemical properties of the product are still intact they view biocide treatment as a needless expense.

3.2.6 Operator time input and the calculated costs per test for CFII

As managers became more wary of relying solely on dip slides the interest in CFII as an alternative monitoring method increased. The largest obstacle in convincing them to fully embrace CFII was the cost per test. As discussed in the previous Chapter there is a trade-off with microbial monitoring methods. Dip slides are inaccurate and unreliable but they are the cheap. Rapid microbiological methods such as CFII can be considerably more expensive but they are also more sensitive, accurate and more informative.

One of the drawbacks of the older Histodenz method was the high demand on operator time and resultant high cost per test. The inclusion of a fluid-handling system with the CFII analyzer facilitated programmable and automated dispensing and sample measurement. In addition to reducing sample variability and human

pipetting errors, the machine and software upgrade have helped reduced overall operator time. The simplified sample preparation method by sedimentation has also greatly contributed to this reduction. The relationship between the number of samples analyzed and the estimated requirement for operator time is shown in Figure 3.13. Dip slides still require the least amount of time to perform: 15 samples can be prepared in 20 minutes and 50 would take 1 hour. Due to the complex and time-consuming steps involved in the Histodenz procedure, with increasing numbers of samples there was also a significant increase in sample preparation time. Testing 20 samples entailed 4 hours of consecutive work from the operator. For a larger plant site with 30-50 samples to be tested daily this value increased to 6-9 hours. This would have provided a large constraint on staff resources since one job position would have been dedicated to product testing alone. CFII with the Histodenz method was not practical and not feasible for use on a large industrial scale. In contrast, the newer sedimentation method was much simpler to perform and required considerably less operator input. Preparing and testing 20 samples took 1 hour and for 50 it was estimated to take 2 hours. These improvements delivered an 80% savings in operator hours. This translates into a savings of 2.5 hours and 7.5 hours per day at a small and large production plant respectively. During the previous NOME trial with the Histodenz method one person's workday was dedicated to analyzing samples on CFII. The sedimentation procedure now frees-up staff resources for other activities and should improve productivity in the quality laboratory.

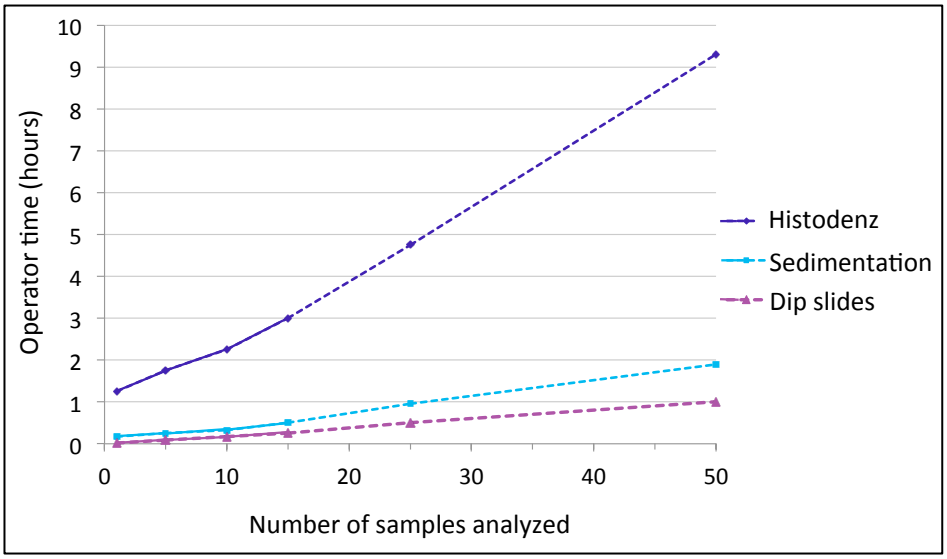
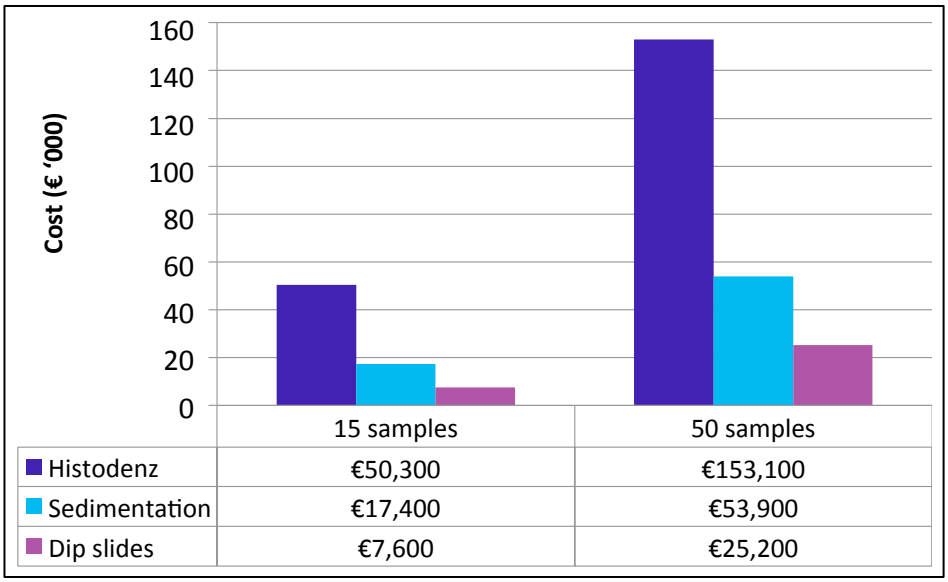


Figure 3.13 - Operator time in hours related to the number of samples analysed each day. The CFII sedimentation method (turquoise) required significantly less input from the operator compared to the old Histodenz method (blue). Dip slides still required the least time to prepare but the CFII sedimentation method could be carried out in a similar timeframe. The solid lines on the graph reflect true recorded times and the dashed lines show estimated times.

The most significant factor in determining the cost per test is the operator time involved. The data from the graph in Figure 3.13 was used in the calculations and a detailed breakdown of the costs is presented in Appendix 1. Assuming NLMJ analyzes 15 samples per day the Histodenz method was estimated to cost €11.10 per sample. The operator time savings achieved from the sedimentation procedure facilitated a 65% reduction in the cost per test to €2.60. When the shipping costs for the consumables and the service contract fees were included this amounted to €4.45 and €12.90 per test for sedimentation and Histodenz respectively. The yearly expenditure was estimated for each microbial monitoring method (Figure 3.14). At NLMJ dip slide analysis could cost the plant €8,000 and implementing CFII by sedimentation would cost €17,500 per year. At a larger Omya site dip slide costs could total over €25,500 while CFII would cost €54,000 per year. Given the estimated €154,000/year to run CFII with the Histodenz sample preparation method, it is not surprising that NOME decided not to undertake regular CFII monitoring at the end of the trial.



	15 samples	50 samples
Histodenz	€50,300	€153,100
Sedimentation	€17,400	€53,900
Dip slides	€7,600	€25,200

Figure 3.14 - Estimated annual costs (€ '000) for each microbial monitoring method. Improvements to the Sedimentation method (turquoise) led to a 65% reduction in CF running costs compared to the previous Histodenz method (blue). Dip slides remained the cheapest testing method.

3.2.7 Summary of pilot study findings

The purpose of the on-site study at NLMJ was to evaluate the performance, ease of use, and costs of different microbial monitoring methods. CFII technology delivered real-time bioburden estimation with high sensitivity and accuracy. Measurements of fluorescence intensity could provide information about the physiological state of the population and the effectiveness of the preservatives in inhibiting microbial growth. Dip slides have long been a standard tool in industry due to their low cost, but they suffer from low sensitivity, are inaccurate and have a 48-hour time-to-result. Previous experience with CFII technology at NOME revealed the Histodenz sample preparation method to be too complex, time-consuming, and expensive. To be considered as a viable alternative to dip slides it was necessary to improve the method. In conjunction with CellFacts 2014 Ltd., a new sample preparation protocol was designed and the instrumentation was upgraded for the pilot study at NLMJ.

Major benefits of the new sedimentation method were:

- The method was straightforward and could be performed by "unskilled" users;
- Most of the sample handling became automated and was carried out by the machine;
- Increased automation facilitated easy scale-up of sample numbers;
- Reduced operator involvement translated to a decrease in the cost per test.

During the pilot study the two CFII methods produced similar results and were able to detect the same trends in population dynamics that dip slides were not sensitive enough to identify. The raw data particle distribution and fluorescence intensity profiles could reveal subtle changes in population growth and the impact of fresh product addition. CFII data also demonstrated that all slurry products possess a baseline level of contamination. QA managers are not microbiologists and they may not appreciate that only a small proportion of microorganisms can be recovered by culturing. For them the concept of a "clear" dip slide is associated with the absence of a microbial population. The CFII results not only uncovered the bioburden in each product, it also showed that different products support different levels of contamination. Also of interest was the revelation that some product grades preserved with AMP have higher microbial loads than when they are preserved with AK. These patterns and differences were not always detectable from dip slide counts. The advantages and disadvantages of the three microbial testing methods are summarized in Table 3.9. Although dip slides provide the cheapest option, CFII and the new sedimentation method became more affordable than previously. It remains the superior method due to its ability to closely monitor a population and provide assistance in biocide management.

Table 3.9 - Comparison of microbial monitoring methods

Assessment criteria	Dip slide	CF Histodenz	CF Sedimentation
Operator time required to prepare samples	✓ 0.3 hours	× 3.0 hours	✓ 0.5 hours
Complexity of sample preparation	✓ Easy	× Challenging, requires training and specialized handling	✓ Simple
Time between sample collection and result	× 48 hours	× 4 hours	✓ 4 hours
Cost per test	✓ € 2.00	× € 11.00	✓ € 4.45
Sensitivity and accuracy	× Low	✓ Very high	✓ Very high
Test gives indication of population viability	× No	✓ Yes	✓ Yes

3.3 Conclusions

Having an awareness of the baseline population in each product allows for trends and any developing contamination problems to be observed in real-time. Action such as biocide addition can be applied more effectively to target the population when it is most susceptible. CFII technology has the potential to direct the timing of biocide addition, inform on the effectiveness of preservation strategies, and help reduce post-production treatment costs. NOME revealed that many customers have negotiated to receive slurry preserved with both AMP and biocide (from discussion

with J. Dyrhaug). As indicated previously some customers request biocide dosing upon unloading into their storage tanks, which cost NLMJ €42,000 in 2014. If this arrangement were part of a customer's consignment deal then it would be difficult to eliminate the service. However, if it were offered as assurance to avoid complaints then it would be possible to phase-out the treatment. CFII measurements taken before a shipment would provide an accurate assessment of bioburden and determine whether precautionary biocide dosing was necessary.

If CFII were applied further upstream at the production site even more cost savings could be achieved. The laboratory manager at NOME identified three areas for cost reduction at their plant: 1) Reduce the concentration of AMP and/or biocide in slurry products using both preservatives; 2) Switch from AMP to monoethanolamine (MEA - a cheaper pH stabilizer); and 3) Reduce the concentration of AMP in some products with good pH buffering (from personal communications with J. Dyrhaug). CFII would be an invaluable tool in these investigations by providing visual and tangible data on the population. The performance of AMP or biocide at lower concentrations could be monitored closely and the impact of the change could be gauged in real-time by examining shifts in the CFII particle and fluorescence profiles.. Dip slides are not be sensitive enough to reveal shifting trends in microbial load and could provide a false sense of security by underreporting TVCs. With respect to replacing AMP with MEA for pH stabilization, which has already been applied at one plant, this poses great risks to maintaining slurry quality and would profoundly alter the microbial community. Biodegradation of MEA has been described in *Achromobacter* sp., *Aeromonas* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Bacillus* sp., *Corynebacterium* sp., *Clostridium* sp., *Enterobacter* sp., *Erwinia* sp., *E. coli*, *Flavobacterium* sp., *Klebsiella* sp., *Micrococcus* sp., *Mycobacterium* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., *Serratia* sp., *Xanthomonas* sp. (Narrod and Jakoby, 1964; Blackwell *et al.*, 1976; Garsin, 2010; Knapp *et al.*, 1996; Hawthorne *et al.*, 2005; Williams and Callely, 1982; Sherburn and Large, 1999). Many of these organisms can use MEA as a sole source of carbon and/or nitrogen, which brings its effectiveness as an antimicrobial into question. Although it is widely used industrially as an ingredient in cosmetics and toiletries, these formulations include other preservatives and biocides (Scholtyssek, 2005; Knapp *et al.*, 1996). Regardless, the application of MEA in slurry would undoubtedly change the microbial community structure and composition, with a strong selection for those organisms capable of utilizing MEA. Given the inclusion of pathogenic organisms in the list above and the potential for organisms to be carried through into finished paper products (Suominen *et al.*, 1997; Vaisanen *et al.*,

1991) a thorough characterization of MEA-stabilized slurry is necessary before considering more widespread usage at other plant sites.

The R&D Microbiology group at Omya is responsible for establishing biocide or AMP/MEA dosage concentrations. MIC testing and product stability is evaluated by culture methods in the laboratory under controlled settings, on a small-scale, and often using monocultures or a mixture of different bacterial isolates from slurry. In reality the microbial community in slurry is complex and dynamic and the response of a mock community will not be able to predict the outcome in an industrial environment. Slurry may be dominated by specific taxa, *Pseudomonas* sp. in AK and alkaliphilic *Bacillus* sp. in AMP (discussed in Chapter 4), but less abundant taxa within populations may also contribute to the development of tolerance and recovery of the population after challenge testing. Thus, the MIC determined for the artificial community in the lab may not exhibit the same effect on a "natural" slurry population *in situ.* Furthermore, it is impossible to predict how community dynamics and composition will shift in response to a new antimicrobial. Culture-based testing of AMP demonstrated its antimicrobial activity on a mock community of *Pseudomonas* sp. and *Methylobacterium* sp. (Di Maiuta, 2010) but did not predict the shift in community structure (discussed in the next Chapter). By working with a natural slurry population during the R&D phase in the lab, antimicrobial activity and adaptations in species abundance could be monitored over an extended time and evaluated more effectively. Instead, AMP proceeded to a pilot-study where dip slide results alone informed on microbial load. In the first few months there were minimal reports of high bioburden levels in AMP products (communications with N. Di Maiuta). An oscillating pattern of a few days of excessive growth (> 10^5 cfu·mL^{-1}) followed by minimal growth (< 10^3 cfu·mL^{-1}) on dip slides was often reported (conversations with N. Di Maiuta and F. Voorbraak). . Samples were sent back to R&D Microbiology for analysis and the few recovered colonies were labeled as "AMP-resistant" or "AMP-tolerant". Since microbial-related product spoilage and perpetually high bioburden levels were not observed, AMP usage was then rolled-out to other plant sites. As will be revealed in the next Chapter, AMP is not a biocide and the "tolerant" species that were identified were common alkaliphilic bacteria. Use of CFII during the pilot study and for daily monitoring at the plants would have shown QA managers the presence of the microbial populations in pH-stabilized slurry. The poor recovery and inconsistent TVCs on dip slides underestimated microbial load and overvalued AMP's antimicrobial activity. In the following Chapter, 16S rRNA gene-based characterization of AMP populations revealed that the current culture media conditions used by Omya do not support the

growth of the adapted alkaliphilic population. This further confirms the inadequacy of dip slides for daily microbial monitoring in slurry.

Despite the many benefits of CFII there was still reluctance at Omya to adopt the technology for daily use. One of the criticisms from NLMJ was that the technology was too sensitive. The bioburden limit stipulated in the product specifications is based on dip slide counts. Since dip slides underestimate microbial load, CF was asked to adapt the algorithm to reduce sensitivity. Furthermore, AMP has enabled better product stabilization even in the presence of microbial loads of 10^5 cfu·mL^{-1}. Many products were regularly flagged close to or above the 10^4 cells·mL^{-1} limit. Adjustments were made to the colour-coding thresholds and "action" points. Efforts were also made to identify the acceptable threshold ranges for contamination in each product. QA managers needed to be able to distinguish between baseline levels of contamination (or those deemed acceptable) and those requiring re-preservation. Each modification of the algorithm and filtering of the raw data excluded informative, good quality data. The method was no longer being used to its full potential. There was some pressure to try and fit the data to a "dip slide-like" result with the true microbial load measurement being suppressed. Ultimately Omya decided that it did not need to employ a method with such high sensitivity. Since the widespread adoption of AMP they have taken the view that as long as the physicochemical properties of the slurry are unaffected by higher microbial load levels, the product specifications limit for bioburden is no longer relevant.

Chapter 4

Characterization of microbial communities in pH-stabilized and biocide-preserved calcium carbonate slurries

4.1 Introduction

Previous characterization studies in slurries were focused on the microbial communities in untreated and biocide-preserved products (Di Maiuta, 2010). The strategy of pH stabilization with AMP was implemented on an industrial scale after successful evaluation in the laboratory. AMP had demonstrated effective antimicrobial activity against *Pseudomonas mendocina* and *Methylobacterium extorquens* isolates from slurry that had developed tolerance to the biocide AK. Preliminary feedback from slurry production plants indicated that the AMP products were stable and microbial-related issues such as high viscosity, acidification, discolouration, and malodour were minimal. Over time reports of high dip slide counts became more common and slurry samples were sent for microbial analysis. Slurry was cultured on TSA and *Bacillus cohnii*, *Brevundimonas diminuta*, and *Ochrobactrum anthropi* were isolated (personal communications with J. Glaubitz). *Pseudomonas* spp. and *Methylobacterium* spp. had always dominated in plate cultures of slurry (Schwarzentruber, 2003; Di Maiuta *et al.*, 2009; Di Maiuta and Schwarzentruber, 2011; Di Maiuta, 2010). The absence of these organisms indicated a shift in the microbial community composition. Furthermore, spore-forming *Bacillus* strains such as *B. cohnii* had never been recovered due to the sporicidal activity of the biocides used. The essential function of AMP was to stabilize slurry at pH 9.5-10.0, while AK slurry was typically maintained at pH 8.5-9.0. It was hypothesized that the microbial diversity in slurry may have changed in response to the more stable alkaline environment. Given the wide range of customers and industrial applications for slurry it was necessary to characterize this new population to ensure it responds to biocide treatment. A multipronged approach was taken to assess microbial load and species diversity in AMP and AK. Slurry samples were received from a production plant and concurrently analyzed using plate culture methods, the RMM CFII (described in the previous Chapters), and by 16S rRNA gene analysis by Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and sequencing on the Illumina MiSeq platform (San Diego, CA, USA).

Traditional culture methods are used in industry for routine viable count assessment and uncovering species diversity in slurry. As discussed in previous sections, some of the shortcomings of cultivation experiments are the strong selective bias imposed on the population and the inability to recover injured cells and dormant cells to a detectable level. Nonetheless, TSA is the one of the standard media for culturing slurry. *Pseudomonas* spp. were the most abundant taxa in AK and readily grew on TSA and PCA media (Di Maiuta, 2010). Di Maiuta

(2010) also observed colony pigmentation on PCA, which can facilitate quick visual assessments of diversity and direct colony selection for species identification by sequencing analysis. However, an important issue to remember is that nutrient rich medium favours organisms with increased growth rate and efficiency of growth. While this is beneficial for microbial load determination within 24-48 hours and is advantageous for the detection of *Pseudomonas* spp., it could influence the composition of the recovered culturable population. Since species differ in their requirements for pH, nutrients, and incubation temperatures, their abilities to recover in culture are also expected to vary. The population in AMP may not respond to the same culture conditions as AK. The microbial communities within slurry have adapted to their environment, which is difficult to replicate in solid media. The variety and concentrations of resources is different and could influence the recovery of some bacteria. While copiotrophic organisms such as *Pseudomonas* spp. can rapidly utilize available resources in high nutrient environments, oligotrophs grow more efficiently in lower nutrient environments. Koch (2001) outlined mechanisms by which the growth and recovery of oligotrophs can be inhibited in nutrient-rich conditions. High nutrient concentrations could impact on the transport of substrates and increase osmotic pressure. The rapid uptake of substrates can also interfere with the concentration of ATP or the proton motive force and lead to energy depletion within the cell. Additional consequences that impair growth include imbalanced cell wall enlargement, dysregulated processes, and the accumulation of toxic substances such as free radicals. The diversity of species that are recovered from slurry in culture may be limited to copiotrophs. Other organisms could be slower growing and outcompeted for nutrients and space or may not be recovered. When culture conditions are severely restricted to, for instance, one type of media, short incubation times, and one growth temperature etc., some taxa will inevitably be over or under represented or absent. By increasing incubation times up to 10 days and culturing slurry on a third medium, TSA adjusted to pH 8.5, it may be possible to increase recovery. In this time the more rapidly growing copiotrophs may have sufficiently reduced nutrient concentrations in the medium to allow oligotrophs to grow.

Microbial load monitoring in industry is dependent on obtaining accurate and reliable viable counts. However, when routine culture conditions do not support the recovery of the most abundant taxa in a sample, bioburden levels would be underestimated and inaccurate. An awareness of the expected community composition helps ascertain the appropriate media and conditions for culture, and this knowledge can be used to determine the limits of detection for specific species.

Whole community analysis of the 16S rRNA gene will help validate the suitability of different media for detecting species composition and abundance in the more alkaline AMP slurry. If inadequate culture conditions are used, false assumptions could be made regarding the efficacy of AMP and susceptibility of the population to biocides such as Rocima 610 (The Dow Chemical Company) (R610). Until community analyses are completed, a more reliable estimation of bioburden levels can be achieved using an RMM based on cell viability such as the CFII system.

As previously mentioned, CFII has been successfully applied in monitoring bioburden and cell viability in slurry (Schwarzentruber, 2003; Schwarzentruber and Gane, 2005; Di Maiuta, 2010; Di Maiuta *et al.*, 2011). CellFacts technology measures the total number of particles in a sample matrix and determines particle size by volume displacement. The technology is able to distinguish viable microbial cells from non-viable particles, based on the degree of uptake of the membrane permeant fluorescent dye $diSC_3(5)$. This probe has been used in studies on red blood cells (Sims *et al.*, 1974) and bacteria (Wu *et al.*, 1999; Zhang *et al.*, 2000) to detect disruptions to the electrical potential gradient across the cellular membrane in response to antibiotics. Similarly, the impact of biocide addition on the contaminating cells in slurry can be observed by monitoring changes in relative fluorescence and shifts in the size distribution profiles. During the pilot-study described in Section 3.2, CFII measurements revealed that different slurry product grades supported different baseline bioburden levels. Another key finding was that identical slurry products preserved with AMP or AK had different microbial loads. Finely ground, pH-stabilized slurry supported a higher degree of contamination, yet its antimicrobial activity against a range of bacteria was demonstrated (Di Maiuta and Schwarzentruber, 2012). The main issue with the early studies of MIC testing of AMP was that it was evaluated in an AK community dominated by *Pseudomonas* sp. adapted to pH 8.5-9.0 (Di Maiuta, 2010). AMP addition would have increased pH above 9.5 and inhibited the growth of a large proportion of the population. With time the community composition and structure could have evolved to include more alkaliphilic species accounting for the higher bioburden levels.

Microbial populations are commonly characterized by analysis of the 16S rRNA gene. 16S rRNA is comprised of highly conserved sequences interspersed with nine hypervariable sequences (V1-V9) that have been exploited for microbial identification, exploring bacterial diversity in different environments, and determining phylogenetic relationships between taxa. Full-length 16S rRNA sequences provide the most accurate taxonomic classification, but methods such as DNA sequencing are limited by shorter read lengths. Various studies have demonstrated the success

of specifying taxa based on gene fragments spanning different hypervariable regions (Huse *et al.*, 2008; Cruaud *et al.*, 2014; Vilo and Dong, 2012; Liu *et al.*, 2007; Liu *et al.*, 2008). Among those investigated, the V1-V3 region was found to be the most suitable for classifying bacteria to the genus level (Petrosino *et al.*, 2009; Kim *et al.*, 2011). However, diversity estimates are prone to biases that could result in the misrepresentation of taxa. The efficiency of cell lysis and recovery in DNA extraction methods can bias the pool of genomic DNA retrieved from the community (Cruaud *et al.*, 2014; Delmont, Robe, Cecillon, *et al.*, 2011). PCR amplification bias from differences in primer binding efficiencies can lead to the preferential annealing of specific templates (Suzuki and Giovannoni, 1996; Pinto and Raskin, 2012). Nucleotide insertions and deletions and the generation of chimeric sequences also contribute to errors during amplification, and the degree of these biases rise with increasing PCR cycle number (Suzuki *et al.*, 1998). Chimeras can arise during PCR from mispriming events or cross-hybridization and their formation has been shown to occur more frequently between closely related species (Haas *et al.*, 2011). Amplification bias against low abundant taxa has been reported where the detection and amplification efficiency of these sequences is reduced relative to high-proportion taxa in the community (Gonzalez *et al.*, 2012). Furthermore, variations in 16S rRNA gene copy numbers in some genomes can also affect relative abundance estimates (Vos *et al.*, 2012). It is important to understand the potential for bias in order to mitigate assumptions and errors during data analysis.

DNA was extracted from the slurry community using direct and indirect methods to uncover bias related to the detection of specific taxa. In direct extraction methods the cells are lysed within the sample matrix and DNA is purified from the cell debris and matrix (Ogram *et al.*, 1987). The indirect approach involves the extraction of cells from the sample environment, commonly by density gradient centrifugation, followed by cell lysis and DNA purification (Courtois *et al.*, 2001). Assuming complete *in situ* lysis of cells, the direct method should achieve higher DNA yields from the community. Harsh physical treatments such as bead beating or sonication have been shown to aid in the release of bacterial cells from soil and sediment microstructures (Robe *et al.*, 2003). Yuan *et al*. (2012) also reported improved cell lysis efficiency and achieved more accurate recovery and representation of a mock community with protocols employing bead beating. While physical methods are capable of disrupting spores and vegetative cells that are more resistant to lysis (More *et al.*, 1994), they are still prone to losing genetic information. The abrasive treatments can result in severe DNA shearing and

released DNA could selectively and irreversibly bind to substances in the sample matrix or be susceptible to chemical degradation (Gabor *et al.*, 2003). Another disadvantage of the direct method is the co-precipitation of contaminants in the sample that can interfere with downstream molecular analyses and reactions. For instance, the presence of clay and aromatic compounds including humic acids and polyphenols found in soils can inhibit PCR amplification and restriction enzyme digestion of DNA (Sagar *et al.*, 2014; Jackson *et al.*, 1997; Lindahl and Bakken, 1995). Direct DNA extraction kits are commercially available and have been widely used in the analysis of microbial communities in soil, sediment, faeces, and the intestine (Williamson *et al.*, 2011; Kennedy *et al.*, 2014; Navas-Molina *et al.*, 2013; Sagar *et al.*, 2014; Delmont, Robe, Clark, *et al.*, 2011). They contain bead tubes and solutions for efficient cell lysis, inhibitor removal, and purification of DNA on columns.

Benefits of indirect approaches include higher purity of extracted DNA and the recovery of larger fragment sizes (Courtois *et al.*, 2001). The centrifugation-based method of cell separation facilitates the removal of extracellular DNA that could bias community composition analyses (Zhao and Xu, 2012). In addition, a proportion of the inhibitors are removed during centrifugation and their concentrations decrease further during DNA isolation (Robe *et al.*, 2003). Since the subsequent cell lysis methods employed are gentler than the physical treatments of direct extraction, the quality and length of the extracted DNA is higher. A major criticism of indirect extraction procedures is the reduced yield observed (Gabor *et al.*, 2003). Chains or clusters of cells tightly associated with particles in the sample matrix could be lost during centrifugation. Di Maiuta (2010) observed the attachment and coverage of *Pseudomonas* sp. on carbonate slurry particles. While an alkaline disruption buffer was used to increase the efficiency of cell dislodgement, it is possible that a proportion of cells remain attached and sediment through the Histodenz. Nonetheless, the combination of increased purity and quality could counterbalance the decreased yield.

Another considerable benefit of the indirect extraction method is the opportunity to limit genetic analyses to the viable cells in a population. Microbial communities are physiologically heterogeneous and will consist of a mixture of active, vegetative and dying or dead cells. DNA is stable and can persist for long periods of time after cell death (Josephson *et al.*, 1993). DNA extraction processes do not discriminate between live and dead cells and studies utilizing PCR amplification could be further disposed to bias. Taxa abundance and community dynamics and turnover can be masked by the inclusion of DNA from dead cells.

The DNA-binding viability dyes ethidium monoazide (EMA) and propidium monoazide (PMA) have been successfully used to block the amplification of DNA in non-viable cells (Nocker *et al.*, 2006; Gensberger *et al.*, 2013; Kobayashi *et al.*, 2010; Nocker *et al.*, 2007; Fittipaldi *et al.*, 2012). Cell extracts are incubated with the membrane-impermeable dyes before DNA purification. The dyes are excluded from live cells with intact membranes but will enter dead cells and those with damaged membranes and intercalate with DNA. Exposure to intense light results in irreversible cross-linking of the DNA and PCR amplification is inhibited. The subsequent DNA extract contains a mixture of amplifiable DNA from viable cells and non-amplifiable DNA from dead cells. EMA or PMA-treated samples generally yield reduced PCR products but produce a more accurate representation of the viable community (Nocker *et al.*, 2006; Fittipaldi *et al.*, 2012).

T-RFLP is a DNA fingerprinting technique commonly used to evaluate the diversity and structure of microbial communities (Siqueira *et al.*, 2010; Osborn *et al.*, 2000; Schütte *et al.*, 2008; De La Fuente *et al.*, 2014; Brugger *et al.*, 2012). It can be a useful tool in monitoring shifts within a population over time or in response to external changes in the environment (Lukow *et al.*, 2000; Dollhopf *et al.*, 2001). A genetic profile is generated based on the size polymorphism of terminal restriction fragments (T-RFs) of amplified near full-length 16S rDNA from the community. Fluorescently labelled primers are used in amplification and the purified products are digested with restriction enzymes. The size and fluorescence intensity of T-RFs are quantified by capillary gel electrophoresis. Peak height in relation to the total measured fluorescence provides an indication of the relative abundance of a given T-RF. Since different lengths of labelled fragments are produced as a result of differences in 16S rRNA gene sequences, each T-RF denotes a distinct operational taxonomic unit (OTU) and the phylogenetic diversity of the community can be inferred. However, the conservation of restriction site positions can lead to identical sized T-RFs for some related organisms so one OTU could represent more than one taxa (Jones *et al.*, 1996). The use of multiple labelled primers and more than one restriction enzyme can overcome this issue by increasing the resolving power (Marsh, 1999; Liu *et al.*, 1997; Schütte *et al.*, 2008). Another issue contributing to the underestimation of diversity by T-RFLP is the reduced ability to detect rare taxa. Species in lower abundance will represent a small proportion of the total community DNA and may not be amplified to a level sufficiently above background noise. These lower fluorescence intensity peaks could be removed during the process of distinguishing true signals from noise. This becomes a greater issue for samples where a lower total fluorescence is measured, indicating that less DNA has been

injected relative to other samples. In theory the concentration of DNA injected should be equal for every sample analyzed and this is ensured in practice by an effective sample preparation method. A final consideration of T-RFLP data is the reported variability within runs (intragel variation between sample replicates) and intergel variation between runs. Identical T-RFs can vary ±1 bp in size and there may be a benefit to extending the size range of the categories (bins) to which fragments are assigned (Osborn *et al.*, 2000; Schütte *et al.*, 2008). Inevitably, this can also contribute to underestimating OTUs and diversity. Although T-RFLP remains an affordable method and valuable tool, amplicon sequencing offers a higher resolution power for taxonomic classification.

Advancements in next generation DNA sequencing technology have helped increase the depth of sequencing and sequence read length, improve accuracy, enable high-throughput processing, and reduce the costs of runs. The Illumina MiSeq platform was selected for its affordability and ability to yield over 30 million sequences of high quality 300 bp paired-end reads (using MiSeq Reagent kit V3). This facilitates the amplification of the hypervariable region V1-V3 of the 16S rRNA gene with a short overlap. The dual-indexing sequencing approach during amplicon library preparation allows for multiplexing of samples on the instrument. Illumina systems have been known to have problems sequencing low diversity samples such as 16S rRNA gene amplicons (Kozich *et al.*, 2013; Fadrosh *et al.*, 2014). Co-sequencing with a control library from phage PhiX artificially increases the genetic diversity in each run and helps yield higher quality reads. The open-source software pipeline called Quantitative Insights In Microbial Ecology (QIIME) is a platform in which various microbial community analyses can be performed (Caporaso *et al.*, 2010). The workflow combines several bioinformatics tools and databases for high-throughput processing of sequence data. Demultiplexing sequences, quality filtering of reads, assigning taxonomy and statistical analyses can all be performed in the QIIME environment.

4.1.1 Aims and objectives

The standard operating procedure at Omya for characterizing slurry dictates culturing slurry diluted 1:10 (in 0.9% w/v NaCl) on TSA media for 48 hours at 30°C. After examination the plate is transferred to 4°C, re-examined after 10 days and then discarded. Colonies may be selected for identification by 16S rRNA gene sequencing or by MALDI-TOF MS. If the slurry sample is from a customer or plant complaining of high microbial load and/or a community displaying resistance to

standard MICs of biocide or AMP then whole community 16S rRNA gene analysis is performed genomic DNA extracted directly from slurry. The aims of the following experiments were to:

- Uncover the limitations of current slurry characterization methods
- Assess the quantitative and qualitative differences in pH-stabilized and biocide-preserved slurries
- Compare the reliability of culture methods with the RMM CFII in measuring viable cell counts in slurry
- Compare community abundance and species diversity results from plate culture and 16S rRNA gene analysis by T-RFLP and amplicon sequencing with respect to:
    - Seasonal changes in community structure
    - Population dynamics in slurry
- Determine the influence of DNA extraction methods on community composition
- Study the impact of biocide addition on the population

Slurry samples were collected and processed as outlined in Figure 4.1.

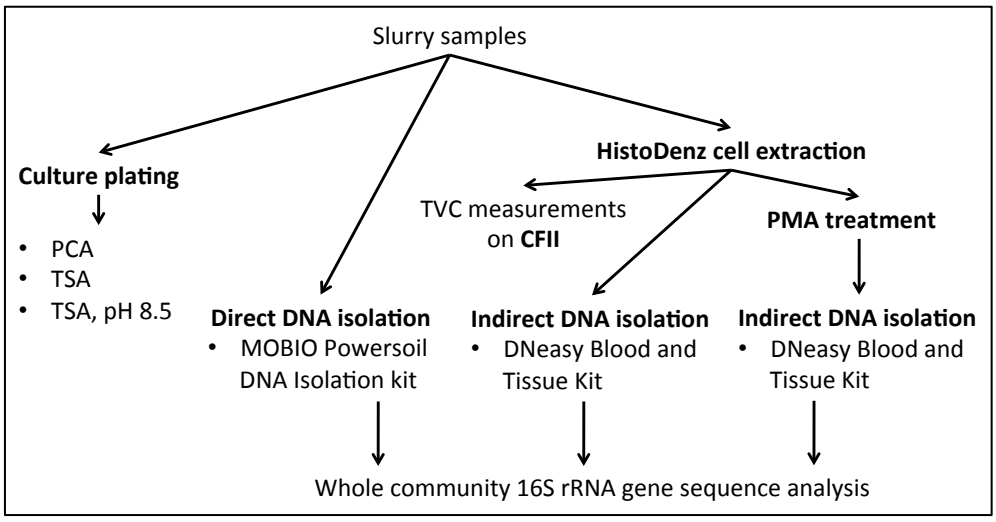


Figure 4.1 - Workflow diagram for slurry characterization.
TVC yields on culture plates were compared to CFII measurements. Species recovery in culture was assessed on three different media and incubated for up to 10 days. The observed community composition was compared with the results of 16S rRNA gene sequences recovered from DNA extracted from slurry. DNA was isolated using a direct method and by an indirect method where cells were first isolated from slurry using Histodenz. Two cell extracts were prepared: one proceeded directly to DNA extraction, and the other was treated with PMA before extraction. The resulting community sequences were analyzed in QIIME.

4.2 Results and Discussion

4.2.1 Microbial load assessment by plate culture

Colony formation was monitored by examining plates after 2 days, 5 days and 10 days. TSA adjusted to pH 8.5 and TSA at neutral pH were the optimal media for assessing TVC after 2 days. Recovery on PCA was slower, counts were often 1-log lower or colonies were very small and difficult to detect. By 5 days of incubation the TVC was equal across all media. The plate culture work revealed that the slurry samples collected in the Summer of 2013 (Su13) of both the pH-stabilized (AMP) and biocide-preserved (AK) slurries were equally heavily contaminated with TVCs above 10^4 cfu·mL^{-1}. In the Spring of 2014 (Sp14) the TVC of AK slurry was in the range of 10^3 cfu·mL^{-1}, whereas Su13 counts were >10^4 cfu·mL^{-1}. During both seasons the TVCs recorded from Week 1 and Week 2 were similar for each slurry type; therefore, there was no apparent change in the culturable microbial load in the tank during one week of storage at the plant.

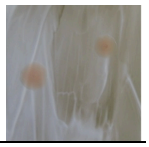
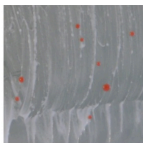
Due to the high density of growth on the plates spread with undiluted slurry, no significant difference in cell counts was observed between Su13 and Sp14 AMP samples. However, mean TVCs of the diluted plate sets showed that the microbial load was higher in Sp14 (4.83E+04 ± 1.53E+03 cfu·mL^{-1}, $P < 0.001$). Samples collected and plated after 48 hours showed an increased TVC but this change was not statistically significant ($P > 0.06$). When AMP slurry was treated with R610, no growth was visible on any media after 5 and 10 days in Su13. In Sp14 a few colonies were observed on plates spread with undiluted slurry collected 4 and 48 hours after treatment and incubated for 5 days (Table 4.1). The colonies were circular, large, pale pink and all identified as *Bacillus cohnii* by 16S rRNA gene sequencing. These surviving cells could have derived from a population of persisters or dormant *B. cohnii* cells that were able to evade biocidal attack. The active proportion of the population is more susceptible to biocide (as discussed in Section 2.3.2.4). These cells quench biocidal activity and decrease the active concentration in slurry to sub-inhibitory levels by reducing the electrophilic groups of R610 (GTA/CMIT/MIT/BNPD). In the absence of additional stress these cells with reduced metabolic activity can return to a more active state and re-establish the population. Another consideration is that *B. cohnii* is spore-forming and this resistant population could have originated from germinated spores. Slurry was not evaluated for the presence of spores in this study. No colonies were detected on plates spread with diluted slurry. This could be due to the dilution of the remaining viable cells to below the limit of detection, or the loss of injured cells from osmotic

stress on the membrane. Nonetheless, biocidal activity was evident from the reduction in microbial load from over 4.00E+04 cfu·mL^{-1} to only a few colonies.

During the analysis of the AK product, the plate culture work indicated that the Su13 samples had a higher microbial load than the Sp14 samples. Prior to sample collection from the storage tank in the spring, the laboratory manager at the plant had reported high dip slide counts and frequent drops in pH in the previous weeks. This trend suggested the presence of the formaldehyde-degrading *M. extorquens* and *Pseudomonas* sp. previously identified in AK slurry (Di Maiuta *et al.*, 2009). The week before shipment the storage tank was dosed with R610 and NaOH to kill the contaminating population and raise the pH above 9.0; therefore, it was unsurprising that the mean TVC was in the low range of 10^3 cfu·mL^{-1} and less than the mean counts recorded for Su13 samples (1.54E+04 ± 6.10E+03 cfu·mL^{-1}, $P < 0.02$). No significant change in TVC was observed between slurry collected and plated after 4 hours and 48 hours in either season. In the R610-treatment group in Su13, no colonies were detected on the plates upon inspection after 5 and 10 days. In the Sp14 R610 group, colonies were visible on the diluted and undiluted plates of samples collected 4 hours after dosing but not 48 hours after (Table 4.1). This would suggest that a contact time of 4 hours was insufficient for achieving gross inhibition or killing of this population at the applied concentration. When placed on media the remaining biocide is diluted allowing the recalcitrant population to recover. No colonies were visible in samples plated 48 hours after treatment; either the population was effectively inhibited with longer exposure to R610 or the conditions were not optimal for awakening dormant cells. The observations of re-growth at the plant confirmed the presence of a persistent population that more readily recovers in the tank. The addition of fresh slurry dilutes the active biocide concentration and the added nutrients facilitate continual growth. In the closed slurry experiments the conditions will not change as dramatically and the possibility of observing growth after biocide treatment is reduced.

The colonies of Sp14 R610 on PCA and most of those on TSA were small, circular, coral pink and identified by 16S rRNA sequencing as *Methylobacterium* sp. (Table 4.1). In the first week, one colony of *Pseudomonas pseudoalcaligenes* was identified on TSA pH 8.5 and a second was detected on TSA in the second week. The lower recovery of *Pseudomonas* sp. suggests that it is more sensitive to R610 than *Methylobacterium* sp..

Table 4.1 - Colonies detected in Sp14 slurry dosed with R610

Sample	Week	Hours after R610 dosage	Colonies observed after 5 days at 30°C (on number of plates out of 3) PCA	TSA	TSA pH 8.5	Species
AMP+R610	1	4	-	One (⅔)	Two (⅓)	*B. cohnii* (right) 
		48	One (⅓)	Two (⅔)	Two (⅔)	
AMP+R610	2	4	One (⅓)	One (⅓)	One (all)	
		48	-	-	One (⅓)	
AK+R610	1	4	Two (⅓)	Two (⅔)	One (⅓)	Predominantly *Methylobacterium* sp. (right) 
		48	-	-	-	
AK+R610	2	4	15-25 (all)	15-25 (all)	-	*Ps. pseudoalcaligenes* also detected (one colony on TSA pH 8.5, Wk1 and on TSA Wk2)
		48	-	-	-	

4.2.2 Microbial load assessment by CFII

Viable counts were determined using the CFII instrument, which calculates TVC using a proprietary algorithm based on the degree of uptake and accumulation of the membrane potential-sensitive probe $diSC_3(5)$. Cells were isolated from slurry using the density gradient medium Histodenz and stained according to the methods outlined in Sections 6.2.5 and 6.2.6. Briefly, slurry was deposited onto a Histodenz cushion and during centrifugation cells were separated from the sample matrix based on differences in buoyant density. Bacterial cells settled within the Histodenz layer and the heavy mineral particles and other debris settled to the bottom of the tube. The supernatant was stained with the fluorescent dyes and the sedimented carbonate material was discarded.

In this set of experiments, CFII was used to measure bioburden in AMP and AK slurry and the effect of R610 addition on the population. At each time of sampling from the plant, AMP slurry was more heavily contaminated than AK (Table 4.2; Su13, $P < 0.001$; Su14, $P < 0.0001$). The CFII viable counts for AMP confirmed that bioburden levels were higher in Sp14 samples (over 10^5 cells·mL^{-1}) than Su13 samples (Table 4.2, $P < 0.002$). For the AK slurry, the CFII counts correlated with the TVCs from the plates in confirming that Su13 slurry supported a higher bioburden than Sp14 slurry (Table 4.2, AK; $P < 0.008$). In both products there was no significant change in the TVCs measured at 4 hours and 48 hours supporting the notion of minimal growth in the population.

Table 4.2 - CFII mean cell counts for summer (Su13) and spring (Sp14) control group samples

Product	Season	Time (h)	Mean TVC (cells·mL^{-1})	SD	*t* test, *P* value		
					4h vs. 48h	Su13 vs. Sp14	AMP vs. AK
AMP	Su13	4	5.33E+04	8.96E+03	0.92	<0.002	**Su13** <0.001
		48	5.27E+04	1.43E+04			
	Sp14	4	1.10E+05	3.14E+04	0.20		
		48	1.35E+05	3.27E+04			
AK	Su13	4	2.29E+04	1.36E+04	0.29	<0.008	**Sp14** <0.0001
		48	1.51E+04	1.01E+04			
	Sp14	4	6.18E+02	4.18E+02	0.16		
		48	1.44E+03	1.27E+03			

Measurements were also taken for slurry after treatment with R610, but a reliable TVC could not be determined. There was no significant difference in the total cell counts of the products between the control and R610 treated groups, which indicated that the cells remained intact and did not lyse. In some instances the mean fluorescence increased after biocide addition. High fluorescence is the result of increased dye uptake. This can be attributed to higher metabolic activity or could be caused by damage to the membrane. Biocides may differ in their targets and mechanisms of action but the end result, a loss of viability, is characterized by depolarization of the membrane and a loss of membrane potential. In theory, this change should be perceived as a decrease in (or absence of) relative fluorescence and a decrease in the calculated TVC. However, if a biocide increases the permeability of the cytoplasmic membrane or cell wall, instead of being excluded the probe may be able to enter the cell, bind to negatively charged residues within the membrane and cause an increase in fluorescence. The CFII algorithm is then unable to distinguish between fluorescence indicating viability and that representing damage to the membrane; thus, the reported TVC is no longer a quantitative measure of viability. Additional consequences of membrane permeabilization are the leakage of cellular constituents and cell lysis. Lysis is detectable by viewing the CFII particle distribution profiles and would be accompanied with a decrease in total cell count. Glutaraldehyde, the main component in R610, results in cross-linking of membrane proteins inhibiting enzyme activity, stabilizing the cell wall and preventing cell lysis (Munton and Russell, 1972). Therefore, R610 treatment would result in an increase in fluorescence but no decrease in cell size and volume.

In AMP samples treated with R610 there was no observable change to the particle-size distribution profiles (Figure 4.2A). The control and R610 profiles overlaid indicating that there was no significant variation in mean cell size ($P > 0.1$) or in total cell count. The fluorescence versus particle size scatter plots also

showed there was minimal change in the relative fluorescence of the cells ($P > 0.4$) (Figure 4.3). The interpretation would be that R610 had a negligible effect on the population; however, we know this to be untrue because no or minimal growth was observed on the plates. In contrast, CFII analysis of AK treated with R610 showed a shift in the particle distribution where the peak cell size increased compared to the control (Figure 4.2B). The mean cell size increased from 0.92 ± 0.01 µm to 0.99 ± 0.01 µm ($P < 0.003$) and mean fluorescence increased ($P < 0.02$) as indicated by the broadening of the scatter density in the fluorescence versus size scatter plots (Figure 4.3). These findings demonstrate the effect of R610 on increasing membrane permeability, leading to a loss in osmotic balance, cell swelling, and increased accumulation of the probe. The responses described for AMP and AK were consistent during each week and in both seasons. Representative sets of the size distribution profiles are presented in Figure 4.2. In addition, the profiles and scatter plots at 48 hours after treatment overlaid those from 4 hours, which signifies the stable and irreversible effect of R610 on the cells.

There are a few explanations for the lack of response of cells in AMP to R610. Firstly, the particle profiles of AMP show the cells in the control samples were already elongated compared to those in the AK controls. Growing cells are elongated so it is possible that a higher proportion of cells in AMP were in a phase of growth compared to cells in AK. On the other hand, AMP was found to cause membrane permeabilization (Di Maiuta, 2010) so the enlarged cells could have been the result of damaged membranes. However, if this were the case then one would have expected to see delayed recovery and growth on the plates combined with low TVCs. Since cultivation experiments routinely observed high bioburden levels ($>10^4$ cfu·mL^{-1}) within 48 hours of incubation it was not likely the cells were injured. Secondly, it was observed previously that the mean fluorescence level was higher in some AMP versions of a product than when it was preserved with AK (Section 3.2.5). During the pilot-study this was attributed to a higher membrane potential from increased physiological activity of the cells. This correlated with the observations of high count and rapid recovery in culture. The absence of a significant change in fluorescence in AMP slurry could have indicated the conditions of the assay were not suited to the environment or population in AMP. CFII recorded a change in the degree of dye uptake when the biocide at the plants switched from formaldehyde-releasers to OPP (communications with C. Dow). This variation likely inferred a physiological change within the cells in response to the new stress. Therefore, it was likely that the cells in the AMP environment were phenotypically and possibly genetically different to those in AK. The mode of action

of AMP is still poorly understood and without knowing the composition of the AMP population it was not possible to predict whether the probes and the assay conditions would be suitable for monitoring AMP slurry.

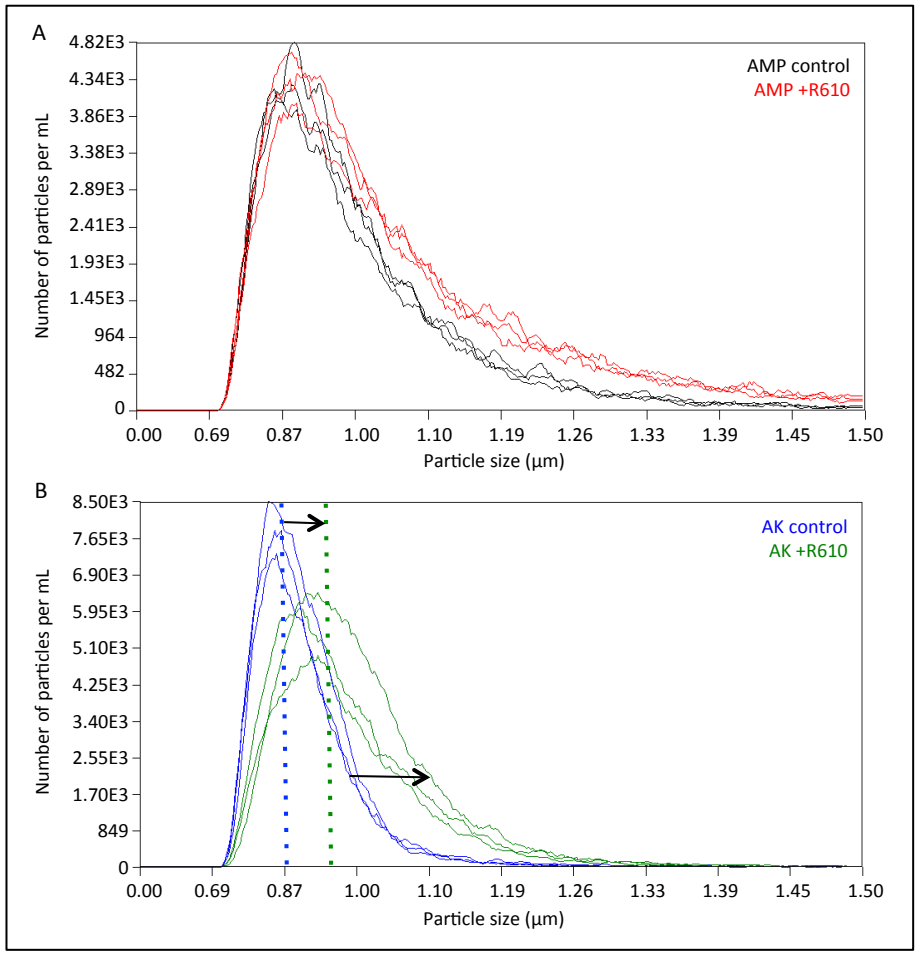


Figure 4.2 - CFII particle-size distribution profiles for slurry with and without R610 treatment.

Samples were collected and analyzed after 4 hours of incubation at 30°C. A) There was no significant change in cell size in AMP slurry after the addition of R610. B) R610 addition to AK slurry caused a global increase in cell size. The peak cell size (indicated by the vertical dashed lines) shifts to the right, suggesting membrane permeabilization and cell swelling. There was no observable change to the size-distribution profiles after 48 hours (data not shown), which signifies the cells were still intact and had not lysed. This same trend was observed in both seasons.

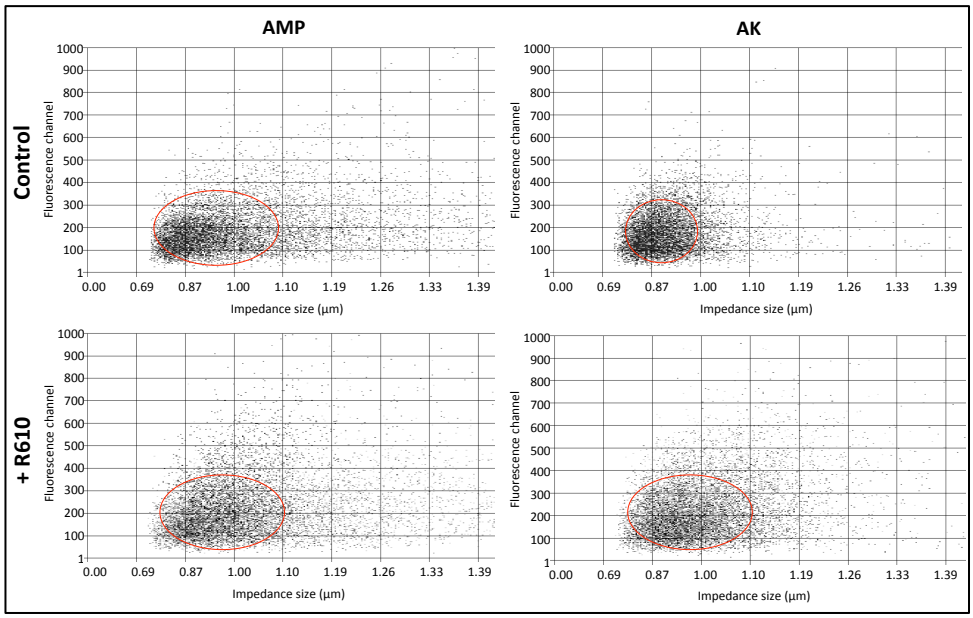


Figure 4.3 - CFII fluorescence versus size scatter plots for slurry with and without R610 treatment.

AMP slurry displayed no significant change in cell size and fluorescence before and after R610 addition (upper left versus lower left plots). The scatter distribution of cells and relative fluorescence remained the same (the density of cells is highlighted by the red ovals). In AK slurry, R610 caused an overall increase in cell size and fluorescence (upper right versus lower right plots). In the control group most cells were clustered within the 0.85-0.98 µm size range (red circle), this area expanded to 0.85-1.12 µm (red oval) after R610 addition.

4.2.2.1 Proposed mechanism of action of AMP

Di Maiuta (2010) equated the mechanism of action of AMP to the antimicrobial activity of other alkanolamines studied in *P. pseudoalcaligenes* (Sandin *et al.*, 1990; Sandin *et al.*, 1992). The study by Sandin *et al*. (1992) showed that uncharged octyl ethanolamine (C_8EA) permeabilizes the membrane causing the leakage of cytoplasmic material. The equilibrium of the unprotonated and protonated forms is maintained with the external environment and both were shown capable of binding to the cell membrane. The authors noted that at high pH conditions the uptake and accumulation of the uncharged form of C_8EA was enhanced and increased cell toxicity was observed. The unprotonated form penetrates the cell membrane and becomes protonated in the proton-rich environment of the cytoplasm. The charged compound becomes amphiphilic, cannot diffuse back across the membrane, and binds to the membrane from within causing a disruption in membrane integrity. The shift in equilibrium resulted in more unprotonated C_8EA being formed externally and further uptake and accumulation in the cytoplasm (Sandin *et al.*, 1992).

The antimicrobial activity of AMP in slurry was investigated by Di Maiuta (2010) and Di Maiuta & Schwarzentruber (2012). Sterile slurry and LB broth were inoculated with *P. mendocina* and challenged with AMP. A common assay for assessing membrane permeability is based on the uptake or exclusion of the hydrophobic fluorescent probe 1-N-phenylnaphthylamine (NPN). NPN is normally excluded from the cell but when membrane integrity is compromised it is able to enter. NPN uptake assays in slurry demonstrated that AMP significantly increased cell permeability and led to the leakage of potassium ions and cellular constituents (Di Maiuta, 2010). Monitoring on CFII also confirmed decreases in average cell size and cell lysis, supporting the mechanism proposed by Sandin *et al*. (1992). Di Maiuta (2010) further proposed that AMP uptake caused the pH within the cell to increase and negatively affect cellular processes. Internal pH above 8.0 causes growth arrest in neutralophiles (Krulwich *et al.*, 2011) therefore AMP was perceived to be a promising antimicrobial preservative. It demonstrated effectiveness as a biocide enhancer by killing a formaldehyde-resistant (AK-resistant) *P. mendocina* in slurry (Di Maiuta, 2010). This organism was capable of growing in media supplemented with 1000 ppm of the formaldehyde-releaser EDDM, where most species exhibit an MIC at 375 ppm (Di Maiuta, 2010). The slurry and broth cultures underwent challenge testing at different concentrations of AMP where a significant reduction in TVC on culture plates was observed. Samples of biocide-preserved slurry from a plant were also dosed with increasing concentrations of AMP and the microbial loads were reduced by 4-log cycles to 10^2 cfu·mL^{-1} (Di Maiuta, 2010). Since *Pseudomonas* sp. were found to be the most abundant genera in biocide-preserved slurry (Di Maiuta, 2010; Schwarzentruber, 2003) the susceptibility of the whole community to AMP was unsurprising. These findings prompted the widespread shift from using biocides to employing a pH stabilization approach.

Previous species diversity work showed that slurry communities were predominated by alkali-tolerant Gram-negative neutralophiles (Di Maiuta, 2010). The pH range of AMP is narrower, more stable, and more alkaline (pH 9.5-10.2) which has caused a shift in the community towards Gram-positive alkaliphiles. Although this was suspected after isolating a few “AMP-tolerant” species from slurry samples from the production plants, it was further confirmed in the cultivation experiments and 16S rRNA gene-based community analyses discussed below. Alkaliphiles possess a higher (more negative) electrical transmembrane potential than neutralophiles (Krulwich *et al.*, 2011) and the lipophilic cationic diSC_3(5) more readily penetrates the membrane of Gram-positive organisms than Gram-negatives (Joux and Lebaron, 2000; Shapiro, 2000). As a result, it is likely the dye

concentration used for cell staining in CFII was too high and the binding sites within the cells had reached saturation. This explains the absence of a change in fluorescence after the addition of R610 to AMP slurry. Since the fluorescence level is no longer informative and CFII cannot provide an accurate estimation of TVCs for AMP slurry, it is no longer a suitable RMM for Omya to use at their plants. Additional testing and optimization of the probes, dye concentrations, and incubation times would be required to restore its functionality and application in pH stabilized products.

4.2.3 Population diversity based on plate culture and 16S rRNA gene sequencing of colonies

TVC determination was demonstrated on TSA and TSA pH 8.5 media within 48 hours, but the colonies were often uniform in colour and in size and did not inform on population diversity. When slurry was cultured on PCA and the incubation time extended to 5 days differences in colony morphology and pigmentation became more apparent. AMP slurry cultured on PCA displayed five colony types in Su13 (Figure 4.4) and three in Sp14 (Figure 4.6). On TSA only two distinguishable colony morphologies were observed in both seasons (Figure 4.4 and Figure 4.6). In AK slurry two distinct colony types were observed on PCA in both seasons compared to only one detected on TSA (Figure 4.4). A representative number of each colony type on PCA and randomly selected colonies from the TSA and TSA pH 8.5 plates were identified by analysis of 16S rRNA gene sequences within the V1-V6 hypervariable regions (as described in Section 6.2.10). 16S rRNA sequences are useful in bacterial classification but its discriminatory power to the rank of species is poor due to the high sequence similarity between some species (Janda and Abbott, 2007). Several isolates obtained sequence identities > 99% for multiple species and it was not possible to conclusively identify organisms to the species level. For instance, *M. extorquens* and *M. populi* share 99.1% similarity in 16S rRNA gene sequences (Van Aken *et al.*, 2004) and *P. mendocina, P. pseudoalcaligenes,* and *P. oleovorans* show > 99% similarity (Mulet *et al.*, 2010; Saha *et al.*, 2010). A number of alkaliphilic *Bacillus* spp. were recovered in AMP slurry and also share high sequence similarities in their 16S rRNA genes, for example, *B. clarkii* and *B. polygoni* (99.5%) (Aino *et al.*, 2008). The phylogenetic relationship of the isolates and closely related species is shown in Figure 4.5. The organisms clustered into three bacterial classes: *Alphaproteobacteria*, *Bacilli* and *Gammaproteobacteria*. *Bacilli* were isolated exclusively in AMP slurry and *Gammaproteobacteria* was

isolated from AK. The species and their colony morphologies observed on PCA are described in Table 4.3.

In AMP the first species to recover in culture was *Brevundimonas* spp., which were present in slurry from both seasons. In Su13 *Brevundimonas* spp. colonies were moderate to large in size (3-5 mm) and were pink, orange, or maroon in colour on PCA and beige on TSA media (Figure 4.4, Table 4.3). This suggests an association between pigment production and medium composition. In Sp14 the colonies on PCA lacked pigmentation and only moderately sized colonies were observed (Figure 4.6). These differences could have been due to the increased cell density in Sp14 slurry. In some species carotenoid pigment production is more visible when colonies were less crowded on culture plates (Khaneja *et al.*, 2010). Given the influence of nutritional and environmental conditions on pigmentation, colony morphology may not be a reliable indicator for the detection of *Brevundimonas* spp. Other colonies detected in Su13 cultures within 48 hours belonged to the genus *Novosphingobium*. Although it was the most abundant species recovered in Su13 AMP and accounted for 59% and 89% of colonies in undiluted and diluted cultures respectively (Figure 4.7), *Novosphingobium* sp. was not observed in Sp14 slurry. The colonies were cream coloured on TSA and yellow on PCA after 3 days of growth (Figure 4.6, Table 4.3) indicating nutrient-dependent pigment production. Yellow colonies also predominated in Sp14 cultures after 48 hours of growth, but pigmentation was observed on all media (Figure 4.6). While 16S rRNA sequence analysis displayed high identities (99%) for *Chryseomicrobium imtechense* and *Psychrobacillus psychrodurans*, the yellow pigmentation restricted its identification to *Chryseomicrobium imtechense* (Arora *et al.*, 2011). This organism represented 91% and 62% of the colonies recovered in undiluted and diluted cultures, respectively (Figure 4.7). The high recoveries and absence of *Novosphingobium* sp. and *Chryseomicrobium imtechense* in opposing seasons represented the largest difference between the AMP samples.

Methylobacterium sp. and *B. cohnii* were isolated from AMP in both seasons. *Methylobacterium* sp. was slower growing, produced small, coral pink/red colonies, and was detected in culture after 5 days of incubation. In AMP this species was only observed on PCA. As previously mentioned, *B. cohnii* was isolated from AMP slurry treated with R610 and the colonies were moderate to large in size and pale pink in colour on all media. R610 inactivated most of the population and the reduced competition for space and resources likely facilitated the increased colony size and pigmentation. In untreated AMP slurry *B. cohnii* colonies were moderately sized (2-3 mm), cream coloured on TSA, and on PCA appeared pink in Su13 and

cream in heavily contaminated Sp14 cultures. Other white or cream colonies found in Su13 and Sp14 were identified as *B. halodurans* and *B. clarkii* respectively (Figure 4.6, Table 4.3). It must be noted that many *Bacillus* spp. have similar colony morphologies on media; therefore, it cannot be definitively stated that a given species was absent in cultured slurry. With colony counts in excess of 10^4 cfu·mL^{-1} only a small proportion of representative colony types were selected for identification and it is likely that species were missed. Perhaps with unlimited time and resources or by use of a rapid colony screening methods such as MALDI-TOF MS (described in Section 2.3.3.3.6) (Welker, 2011; Biswas and Rolain, 2013; Sandrin *et al.*, 2013), every colony could have been selected for analysis.

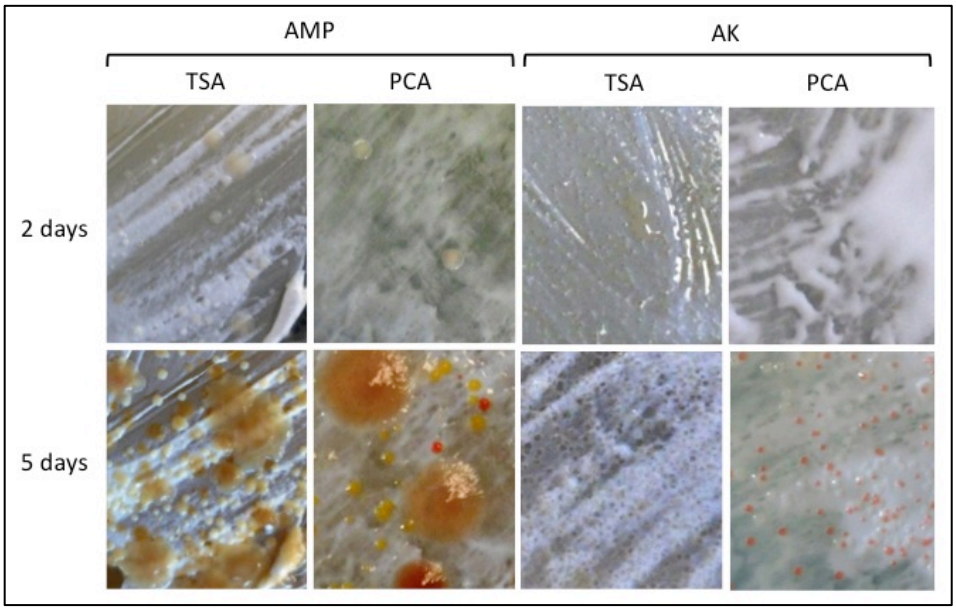


Figure 4.4 - Growth of Su13 AMP and AK slurry samples on TSA and PCA media.
Representative pictures of one AMP and one AK undiluted slurry samples plated on TSA and PCA. After two days of incubation (top panel) colonies were readily visible on TSA. *Brevundimonas* sp. and *Novosphingobium* sp. were detectable in AMP and *Pseudomonas* sp. was observed on TSA in AK slurry. A more accurate TVC count was obtained after 5 days when slower growing and slow to recover colonies appeared (bottom panel). Species distinction is more apparent on PCA due to colony pigmentation. In AMP slurry, the larger pale pink, orange, or maroon colonies were identified as *Brevundimonas* sp., the yellow colonies were *Novosphingobium* sp., white colonies were *B. halodurans*, pink colonies were *B. cohnii*, and the small coral pink colonies were *Methylobacterium* sp. (Table 4.3). AK slurry also contained *Methylobacterium* sp. (discernible only on PCA) and the cream/beige colour colonies on PCA and TSA were identified as *Pseudomonas* sp. (Table 4.3).

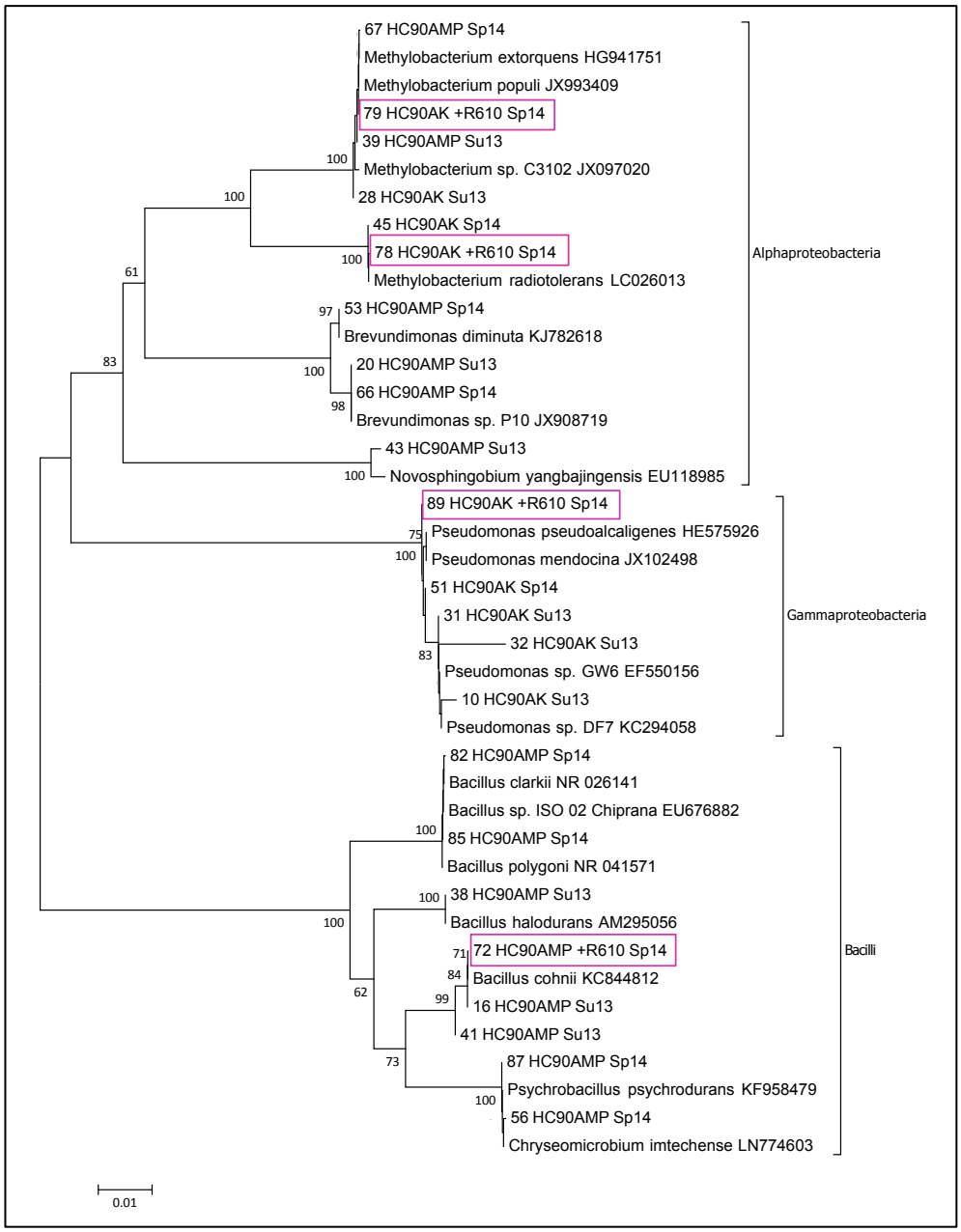


Figure 4.5 - Phylogenetic tree constructed from the partial 16S rRNA sequences showing the position of selected colony isolates and closely related species.

Pink boxes highlight the colonies isolated from R610-treated slurry. Accession numbers for the related species are found after the strain name. Phylogeny was inferred using the Neighbour-joining method. Evolutionary distances were computed using the Maximum Composite Likelihood method and bar length equates to the number of nucleotide substitutions between sequences. The bootstrap test (1000 replicates) was used to estimate standard error; the values over 50 are shown next to the branches. Analyses were conducted in MEGA6 (Hall, 2013).

Table 4.3 - Isolates identified in AMP and AK slurry based on colony morphology on PCA

Closest related species	Colony morphology (Form, size, elevation, margin, opacity, surface, pigmentation)	Pigment development (days)	HC90	Season	
				Su13	Sp14
B. cohnii	Circular, moderate, low convex, entire, opaque, smooth, cream or pink	NA or 5	AMP	✓	✓
B. halodurans	Circular, moderate, low convex, entire, opaque, smooth, cream	NA	AMP	✓	
B. clarkii/B. polygoni	Circular, moderate, low convex, entire, opaque, smooth, cream	NA	AMP		✓
Brevundimonas sp. P10; *Brevundimonas diminuta*	Circular, moderate to large, convex, entire, opaque, glistening, cream white, pale pink, orange or maroon	3	AMP	✓	✓
Chryseomicrobium imtechense	Circular, moderate, low convex, entire, opaque, smooth, yellow	2	AMP		✓
Novosphingobium sp.	Circular, moderate, convex, entire, opaque, smooth, deep yellow	3	AMP	✓	
M. extorquens/M. populi; *M. radiotolerans*	Circular, small, convex, entire, opaque, smooth, coral pink or red	5	AK/AMP	✓	✓
Ps. pseudoalcaligenes/Ps. mendocina/Ps. oleovorans	Circular, moderate, raised, undulate, opaque, glistening, beige	NA	AK	✓	✓

NA=Not applicable

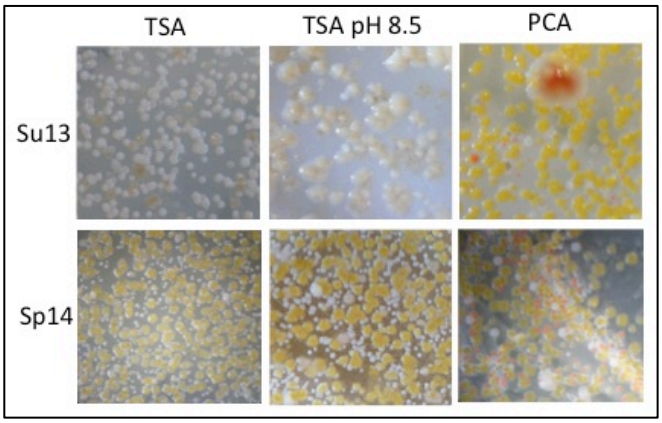


Figure 4.6 - Colony morphologies of diluted AMP slurry from Su13 and Sp14 cultured on media for over 5 days.
Top panel: Su13 AMP slurry displayed minimal distinction between colony types on TSA and TSA pH 8.5, but differences in colony morphologies were visible on PCA. Species were characterized as described in Figure 4.4 and Table 4.3. Bottom panel: In Sp14, colony types and pigmentation were similar on TSA and TSA pH 8.5, but unlike in Su13, yellow-pigmented colonies were recovered and identified as *Chryseomicrobium imtechense*. *S*mall, pink colonies belonging to *Methylobacterium* sp. were also visible. The selected white/cream colonies were identified as *Brevundimonas* sp. (which did not display the same pink/maroon colouration observed in Sp14) and *B. clarkii*.

Fewer species were isolated from AK slurry than from AMP. The cream/beige colonies visible within 48 hours belonged to the *Pseudomonas* genus (*P. pseudoalcaligenes*, *P. oleovorans*, or *P. mendocina*) (Figure 4.4). After 5 days of culturing on PCA, small coral colonies of *Methylobacterium* sp. (corresponding to *M. extorquens* and *M. populi*) and *M. radiotolerans* were recovered (Figure 4.4). *Pseudomonas* sp. dominated on TSA and likely outcompeted *Methylobacterium* spp. for nutrients and space. The only instance where *Methylobacterium* spp. were detected on TSA was in AK samples treated with R610 (Table 4.1). *Pseudomonas* sp. was inhibited more rapidly, which may have allowed surviving *Methylobacterium* spp. to recover on TSA. Cultivation experiments indicated the culturable population in AK slurry did not differ between seasons.

4.2.3.1 Cultivation experiments to determine species abundance

Incubations times of 5 days were required to maximize the detection of the culturable population and to achieve a more accurate depiction of relative species abundance. Colony morphology and 16S rRNA gene sequences were used to determine species distributions on PCA. Differences were observed between undiluted and diluted slurry cultures where species in lower abundance were generally more readily detected on undiluted plates. For example, *B. cohnii*, *B. halodurans*, *Methylobacterium* sp. and *Brevundimonas* sp. constituted 40% of the

colonies in undiluted cultures and represented only 12% of detected colonies in diluted samples (Figure 4.7). The main advantage of culturing neat slurry is the increased ability to detect more taxa, including those in low abundance and organisms with a lesser capacity for growth on nutrient media and slower growth rates. In some instances the opposite effect was observed where *B. clarkii* and *Methylobacterium* sp. colonies were not detected in undiluted Sp14 AMP cultures but were recovered in diluted cultures (Figure 4.7). These inconsistencies could be attributed to variations in the responses to culture conditions, the level of competition between individual community members, and the differing physiological states of cells.

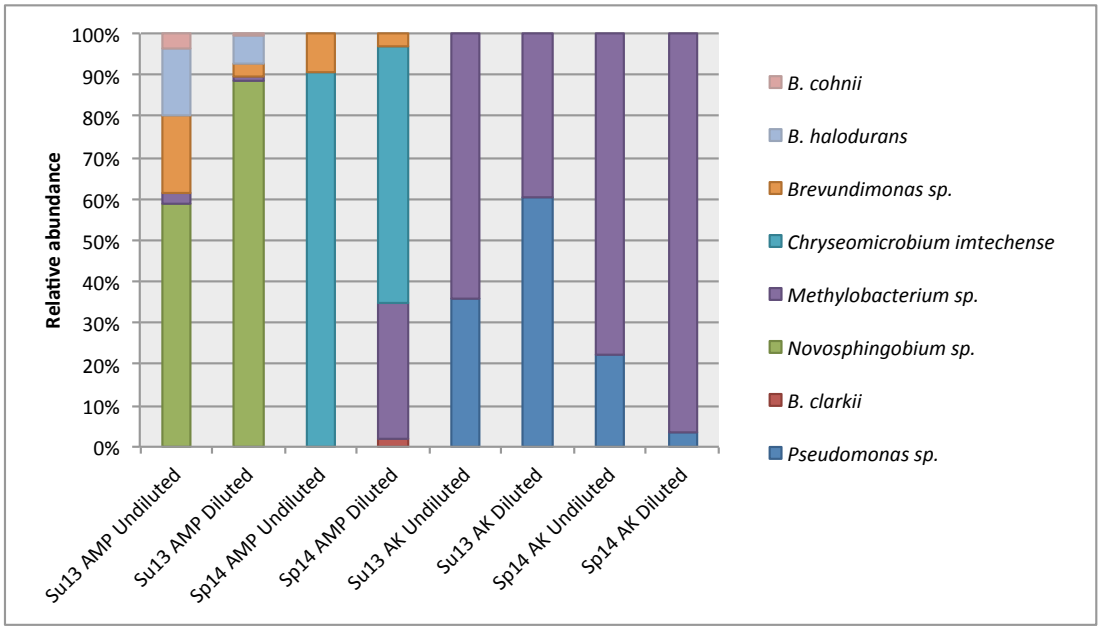


Figure 4.7 - Mean relative abundance of species in undiluted and diluted slurry on PCA after 5 days.

Species were identified by 16S rRNA sequencing and abundance was inferred by counting based on colony morphology. Data is shown for the first week's samples collected from the plant. The mean relative abundance was similar on triplicate plates and between the 4 and 48 hours sample collection points.

As previously mentioned, the high viscosity and opacity of slurry makes it difficult to spread thinly and evenly over the agar surface and can hinder colony visibility. Diluting slurry enhances detection and should also aid in alleviating competition by reducing cell counts in cultured samples. In addition, the diluent 0.9% w/v NaCl provides additional salts that could benefit some organisms over others. In certain instances some organisms may favour the carbonate environment and the buffering capacity provided in undiluted slurry cultured on pH neutral media. This could be particularly important for minimizing pH shock in the AMP community,

which grows in alkaline conditions above pH 9.5. In undiluted cultures nutrients from the media are still accessible and the slurry matrix could also help reduce the impact of nutrient shock to oligotrophs within the population.

Azevedo and colleagues (2012) evaluated the recovery of bacterial test strains grown in low and high nutrient environments. The authors monitored the average improvement in culturability when these organisms were subsequently grown on nutrient poor and rich media. After adaptation to low nutrient conditions the organisms displayed better recovery (higher CFU) on lower nutrient medium, and the reduced growth observed on rich-nutrient medium was attributed to nutrient shock. In another study, Song *et al.* (2015) investigated the effect of nutrient concentrations and incubation times on the recovery and composition of a bacterial community in soil. Samples were cultured on undiluted medium (TSA 1/1) and diluted media ranging from TSA 1/10-1/10000. Across all media the concentration of nutrients was highest in the early stages of incubation and gradually decreased as biomass accumulated over time. Cultures on diluted TSA media showed higher species diversity and exhibited a shift in community composition that was not observed on TSA 1/1. Copiotrophic taxa dominated in the earlier time points and subpopulations of oligotrophs appeared as the availability of nutrients decreased. Common copiotrophs observed at all nutrient concentrations belonged to family *Pseudomonadaceae* and phylum *Firmicutes*, and genus *Sphingomonas* was among the oligotrophic taxa detected in high abundance in older cultures (Song *et al.*, 2015). Another key observation was that the greatest diversity achieved on all nutrient concentrations was exhibited after 56 days of incubation. It was speculated that intermediate nutrient concentrations were reached that supported the coexistence of copiotrophic and oligotrophic taxa.

In the context of culturing slurry, a similar trend could be expected where the dominant organisms recovered are limited to copiotrophs. Given the short incubation times and use of nutrient rich media the detection of oligotrophs is less likely. Indeed, in AMP slurry species that recovered rapidly included *Brevundimonas* spp., *Novosphingobium* sp., and *Chryseomicrobium imtechense*. *Methylobacterium* sp. and *Bacillus* spp. were detectable after increased incubation and generally produced fewer numbers of colonies (Figure 4.7). On the one hand this could indicate that the latter organisms are present in lower abundance in slurry; on the other hand, their slower growth rate and low numbers could also be ascribed to competitive exclusion by the faster growing species or inhibited growth under inadequate culture conditions. Alkaliphilic *Bacillus* strains grow optimally around pH 9.5 (Takami, 2011). Although slurry was additionally cultured on TSA at pH 8.5, a

higher proportion of colonies were isolated and identified from PCA due to the visible distinction of species by colony morphology. On pH neutral media there is an increased possibility of osmotic shock to cells adapted to the alkaline slurry environment. The growth efficiency and recovery of *B. halodurans*, *B. cohnii*, and *B. clarkii* on PCA may have been inhibited and their abundance in slurry was likely underestimated. The optimal growth conditions for the organisms isolated from AMP and AK are listed in Table 4.4. With the exception of the mentioned *Bacilli*, the isolates all grow optimally under the employed conditions (neutral pH and incubation at 30°C). For enhanced recovery and improved competitiveness of alkaliphiles over neutralophiles, a higher pH and higher salinity medium may be required and a range of growth temperatures should be tested.

In AMP samples received in the following week no change in TVC was recorded, but significant differences in the proportional abundance of some species was seen. In diluted Su13 samples the proportion of colonies of *Novosphingobium* sp. decreased from 89% to 63% ($P < 0.005$) and those of *Brevundimonas* spp. and *B. halodurans* increased over 6.5- and 1.5-fold respectively ($P < 0.01$) in the second week of sampling. Sp14 samples displayed a similar shift where the dominant *Chryseomicrobium imtechense* decreased in proportion from 62% to 46% ($P < 0.05$) and colonies of *Brevundimonas* spp. increased 3-fold and *Methylobacterium* sp. 1.3-fold ($P < 0.01$). Keeping in mind the limitations of cultivation methods, these changes in abundance could have been influenced by the differing physiological states of the cells at each time of culturing. The growth rate and ability to out-grow competitors varies during different phases of growth. The mixed microbial community of slurry contains different subpopulations of cells; therefore, shifts in the abundance of species recovered in culture would be expected from week to week. Differences were also anticipated between the seasons. External environmental conditions during rock extraction, the microbial community in water sources, the ambient temperature in the storage tanks, and the cleanliness of contact surfaces in the plant change over time and all impact upon the innate contaminating community.

Table 4.4 - Additional characteristics of species isolates

Closest related species	pH range (optimum)	Temperature range (optimum) (°C)	Physiological characteristics	Spore-forming	References
B. cohnii	8-10 (9)	10-47 (37)	OA	Yes	Spanka & Fritze (1993), Yumoto *et al.* (2011)
B. halodurans	7-10.8 (9-10)	15-55 (37)	FA	Yes	Nielsen *et al.* (1995), Yumoto *et al.* (2011)
B. clarkii/B. polygoni	8-11 (9-10)	15-45 (29-39)	OA	Yes	Aino *et al.* (2008), Nielsen *et al.* (1995), Yoshimune et al. (2010)
Brevundimonas sp. P10; *Brevundimonas diminuta*	6-8 (7)	25-37 (28-30)	NP	NA	Segers *et al.* (1994)
Chryseomicrobium imtechense	6-9 (7)	4-45 (30)	NP	NA	Arora *et al.* (2011)
Novosphingobium sp.	6.5-8 (7)	15-37 (30)	NP	NA	Sohn *et al.* (2004), Suzuki & Hiraishi (2007)
M. extorquens/M. populi; *M. radiotolerans*	6-8 (7-7.4)	20-30 (28)	NP	NA	Urakami *et al.* (1993), Van Aken *et al.* (2004)
P. pseudoalcaligenes/P. mendocina/P. oleovorans	6-10 (7)	15-45 (35-37)	NP	NA	Gavini *et al.* (1989), Saha *et al.* (2010)

FA=Facultative alkaliphile; NP=Neutralophile; OA=Obligate alkaliphile

While AMP slurry demonstrated changes in the dominant culturable species that were detected, this population was unchanged in AK in both seasons. *Methylobacterium* spp. were generally observed in higher abundance on PCA than *Pseudomonas* sp. (with the exception of diluted Su13 samples) (Figure 4.7). *Pseudomonas* sp. may have represented a lower proportion of the population in Sp14 due to the repeated dosing of R610 at the plant, which was required to control the high microbial load in the product. The subsequent R610 treatments in these experiments revealed that *Pseudomonas* sp. was more susceptible to R610, and a subpopulation of *Methylobacterium* spp. were able to survive preliminary exposure to recover in culture. Indeed, microbial populations in biocide-preserved slurry are often challenging to control and biocide-tolerant communities have been identified (Schwarzentruber, 2003). Di Maiuta (2010) isolated *Pseudomonas* spp. (including *P. mendocina*, *P. putida*, *P. pseudoalcaligenes*) and *Methylobacterium* spp. (*M. extorquens* and *M. radiotolerans*) in recurring biocide-tolerant populations. In another study Di Maiuta *et al.* (2009) described a community of *M. extorquens* and *P. putida* that alternated in abundance in culture. Fluctuations in species dominance were linked to residual formaldehyde concentration in slurry. When concentrations were high *M. extorquens* was recovered in greater abundance in plate cultures; conversely, *P. putida* dominated when formaldehyde concentrations were found to be low. It must be noted that these results were not confirmed by quantitative genetic analyses of the population. Although formaldehyde concentrations correlated with culturability, it is possible that the changes in abundance were not reflected in the slurry population.

4.2.4 Characterization of microbial diversity by 16S rRNA gene analysis

4.2.4.1 Indirect and direct DNA extraction methods

DNA was extracted directly from slurry with the Powersoil® DNA Isolation kit (MO-BIO), a kit validated by Di Maiuta (2010) for use with slurry. Two methods for indirect DNA extraction were used. First, two cell extracts for each sample were prepared using the Histodenz method discussed previously. One extract proceeded to DNA isolation using the DNeasy® Blood and Tissue Kit (QIAGEN). The second extract was treated with the viability dye PMA before continuing on to isolation with the DNeasy kit.

4.2.4.2 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

In this study community fingerprints of the slurries were to be compared to previous work by Di Maiuta (2010), thus the same methodology and experimental procedures needed to be performed. Universal bacterial primers EUB8m-f (Weisburg *et al.*, 1991) and EUB1088-r (Lee and Kemp, 1994) were used to target the hypervariable regions V1 to V6 of the 16S rRNA gene (Section 6.2.10). Only the forward primer was labelled with 6-Carboxyfluorescein-labelled (6-FAM) and the 1100 bp PCR products were digest with the restriction enzyme *MspI*. The amplification yields varied according to the extraction method used and the slurry treatment group. For instance, amplification was poor from DNA from slurries treated with R610 and extracted from PMA-treated cells. If biocide treatment was successful, there should be few viable cells remaining; similarly, successful interaction of PMA with DNA in non-viable cells should have inhibited PCR amplification. The data normalization method is outlined in Section 6.2.11.2 and diverges from the method applied by Di Maiuta (2010), which follows the iterative standardization procedure recommended by Dunbar *et al.* (2001). This modification was necessary to accommodate for the large discrepancies in total fluorescence observed across all the samples. When the iterative process of normalizing to the smallest total peak height was applied, abundance data was skewed toward the dominant T-RFs and less abundant OTUs were subsequently lost. Instead, samples were standardized by calculating the relative abundance of each T-RF according to their contribution to the total fluorescence peak height. T-RFs contributing to less than 1% of the total were removed and the relative abundance was recalculated. Another deviation from Di Maiuta (2010) was the inclusion of triplicate biological samples. To generate a reproducible derivative profile the replicate profiles were combined as described by Dunbar *et al.* (2001).

To assist in the identification of T-RFs, DNA extracts of the species isolated from culture also underwent T-RFLP analysis. Clear, distinct T-RF peaks were visible for each organism (Table 4.5). In some instances it was not possible to associate the T-RF to a specific size. According to Dunbar *et al.* (2001) T-RFs that differ by greater than 0.5 bp are two distinct peaks, and those differing by less than 0.5 bp are identical and can be clustered. For example, due to slight variations in the run, the T-RF size for *B. cohnii* was observed at 152.6 ± 0.2 bp. To correct the placement of *B. cohnii* T-RFs into separate bins, the T-RFs were redefined as a split between 152-153 bp. This adjustment was also applied for other isolates (refer to Table 4.5). It is important to recall that other taxa could possess the same T-RF size. The recovery of these organisms in culture implies their presence within the

culturable fraction, but the relative abundance indicated by T-RFLP could be overestimated and the contribution of other organisms could be masked. Still, this knowledge provides some context during the interpretation of T-RFLP profiles.

Table 4.5 - Measured T-RFs of species isolated from slurry

Organism	Measured T-RF size (bp)
Chryseomicrobium imtechense	144
Novosphingobium sp.	146-147
Methylobacterium sp.	149
B. cohnii	152-153
B. clarkii	156-157
B. halodurans	162
Brevundimonas sp.	401
Pseudomonas pseudoalcaligenes	489

4.2.4.3 Illumina MiSeq 16S rRNA gene sequencing

A dual-indexing approach was developed from modifications to the 16S metagenomic sequencing library preparation method from Illumina (Illumina, 2013). Three PCR reactions were required to generate the final product to be analyzed on the MiSeq (refer to Section 6.2.12.1). First, the V1-V3 hypervariable region of the 16S rRNA gene was targeted to encompass the same region from which the T-RFs were generated. The same forward primer, EUB-8mf, was used with the reverse primer EUB-515r (Baker *et al.*, 2003) and together amplified a product around 520 bp. This initial amplification step was required to increase the low concentration of template DNA in many samples. The second PCR facilitated the addition of the spacer sequences known as “Read 1” and “Read 2” onto which the final adapters were added during the third PCR. These adapters contain an 8 bp index sequence and the linker sequences P5 and P7 that bind to the flow cell and initiate sequencing. Seven forward and twelve reverse indexed-primers were applied in unique combinations to individual wells of a multiwell plate. Each plate and subsequent MiSeq run contained 79 distinct PCR products for sequencing. Every plate contained technical replicates to evaluate the variability that arises during library preparation or the sequence run. A mock community control was also included in each run to determine the inter-run variability. The mock community was prepared from a mixture of amplified 16S rDNA from the species isolates. One control sample was prepared and this was split equally among the four libraries.

During the run itself a control library is always added to assess the quality and success of the run. The number of reads for each run exceeded 20 million; the run summaries are listed in Table 4.6. The raw data sequence files were obtained

and processed as outlined in Section 6.2.12.3 following the QIIME 8.0 pipeline (Caporaso *et al.*, 2010). Briefly, the index reads were combined and the barcode information was used to merge the respective forward and reverse sequences. At this stage a proportion of reads were lost due to the removal of sequences with mismatched or missing barcodes and those corresponding to the PhiX library. During the demultiplexing step the compilation of all the sequences was separated and assigned to each respective sample. Applying a high Phred quality score (Q>30) at this stage increases the confidence level and accuracy of the retained sequences. The Phred score denotes the probability that a given base call is incorrect, and the higher the Q-score the more reliable the call. Each base is evaluated and sequences were truncated at bases with low Q-scores. If the resulting sequence was too short then the read was discarded. Errors can be introduced during each PCR stage of library preparation and in the sequencing runs themselves. Conservative quality filtering and the inclusion of erroneous reads can lead to difficulties in assigning taxonomy and can overestimate diversity. Although only 20-67% of the successfully merged reads were retained after filtering (Table 4.6), an abundance of data still remained.

Table 4.6 - MiSeq run summary and number of reads after data processing and quality filtering

Run	Raw sequence reads				Reads after quality filtering	
	Cluster density (10^3/mm^2)	% Of bases ≥Q30	Reported machine error (%)	No. of reads passing filter (M)	Successfully merged paired ends (M)	Q>30 (M)
1	1176 ± 55	66.0	1.20	27.1	10.7	4.4
2	888 ± 75	65.7	1.31	20.4	8.5	2.0
3	1106 ± 73	70.7	0.93	27.0	12.3	8.2
4	1248 ± 68	65.5	1.26	29.2	9.3	1.9

To process the data more efficiently, the demultiplexed data from the four runs were combined and processed together. Reads were aligned to pre-aligned Greengenes 16S rRNA gene sequences (13_08 release) at 97% identity (DeSantis *et al.*, 2006; Werner *et al.*, 2012). At this stage a consensus sequence of the overlap region was generated from the paired-ends. Reads failing to overlap because of quality truncation were stitched together, which could introduce bias during taxonomy assignment (Fadrosh *et al.*, 2014). Sequences were assigned to taxonomic rank using the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007). When the UCLUST method (Edgar, 2010) was used, 7% of the sequences could not be assigned, compared to only 0.4% with the RDP classifier. The compiled OTU table was filtered to remove chimeric sequences and OTUs observed

fewer than two times. The table also filtered out OTUs with sequences representing less than 0.005% of the total sequences as recommended by Bokulich *et al.* (2013). The number of OTUs generated before and after filtering is shown in Table 4.7. This was considered necessary to reduce the impact of low abundance OTUs in evaluating the statistical significance between communities in different groups. For the 316 samples there were 13,695,134 sequences for analysis. Despite normalization efforts there was a large range of sequences per sample (median=36,223, *M*=43,339, *SD*=35,901). DNA extracts from the biocide treated group had lower concentrations of template DNA and it was difficult to amplify product. These samples tended to have the lowest counts, still, only 6% of samples had below 2200 sequences/sample. Wherever possible the highest sampling depth was applied in analyses in order to incorporate the greatest number of samples.

Even with filtering, the abundance of OTUs is still likely overestimated. For instance, theoretically the mock community should have contained eight OTUs, but 13 were identified: 4 *B. cohnii*, 2 *B. halodurans*, 2 *Novosphingobium,* 1 order *Bacillales*, 1 *Bacillaceae* family, 1 *Caulobacteraceae* family, 1 *Methylobacterium* sp., and 1 *Pseudomonas* sp.. The generation of multiple OTUs for a single isolate could be the result of PCR or sequencing error. Another consideration is the copy numbers of 16S rRNA genes within bacterial genomes. *Firmicutes* and *Gammaproteobacteria* have been shown to possess 5.8±2.8 copies of the 16S rRNA gene per genome and display high within-genome variability (Větrovský and Baldrian, 2013). Variability in copy numbers was also high at the level of families, including *Bacillaceae* and *Pseudomonadaceae,* and in some genera. This is an important factor to keep in mind when quantifying and estimating diversity. Another interesting observation from the mock community analysis was that organisms such as *B. clarkii*, *Brevundimonas* sp. and *Chryseomicrobium imtechense* could only be classified to the level of order or family. In their study, Větrovský & Baldrian (2013) also found that in 12.2% and 41.7% of genera, the similarities of 16S rRNA genes among species were higher than 99% and 97%, respectively. Species identification of the colony lysates was supported by the observed colony morphologies; however, when analyzing the whole community it may not be possible to resolve these organisms to the species level. This would decrease estimates of diversity if other organisms are similarly restricted to clustering to an order. Another possibility that could impact on taxonomy assignment is the degree of truncation of sequences during quality filtering. There may be insufficient variability to direct OTU clustering at the 97% similarity level.

4.2.4.4 Microbial community composition

4.2.4.4.1 Overview of species diversity in AMP and AK slurry

The cultivatable population of AMP slurry was demonstrated to be more diverse than the population in AK. T-RFLP analysis revealed 17 OTUs in Su13 AMP slurry and 14 in Sp14 (Table 4.7). Analysis of variance (ANOVA) revealed there was no statistical significance ($P > 0.05$) in the mean number of OTUs observed between the seasons. Five of these OTUs were detected during both seasons: 152bp, 162bp, 164 bp, 401 bp, and 553 bp (Figure 4.8). The MiSeq sequencing results indicated a higher number of OTUs. After quality filtering of the OTU table, 94 and 111 OTUs were observed in Su13 and Sp14, respectively (Table 4.7). In Su13 AK slurry 14 OTUs were detected by T-RFLP and 98 OTUs were estimated by sequencing analysis; these numbers increased to 21 and 244 in Sp14, respectively (Table 4.7). The most frequently observed T-RF in AK was the 489 bp OTU. The other two OTUs present in both seasons were 114 bp and 268 bp, but they were only observed in a small number of samples (Figure 4.8).

Table 4.7 - Number of OTUs based on T-RFLP and sequencing of 16S rRNA genes

Slurry	Su13			Sp14		
	T-RFLP	MiSeq		T-RFLP	MiSeq	
		Before quality filtering	After quality filtering		Before quality filtering	After quality filtering
AMP	17	128	94	17	147	111
AK	14	242	98	21	553	244

Principal Component Analyses based on Jaccard distance was carried out on the T-RFLP abundance data to compare the relationship between the samples (Figure 4.9). Component 1 (x-axis) accounted for 24.7% of the variance and the AK samples from both seasons were distinctly separated from AMP. The dominant T-RF in AK at 489 bp resulted in Su13 and Sp14 samples to group together along component 2 (y-axis). The second component accounted for 14.1% of the variance and showed a seasonal-dependent separation of the AMP samples (Figure 4.9). Two groups were discerned based on the differences in the T-RFs detected in AMP during each season. Cluster analysis based on Bray-Curtis distance was performed on the T-RF data and also showed distinct clustering of AK samples from AMP (Figure 4.10).

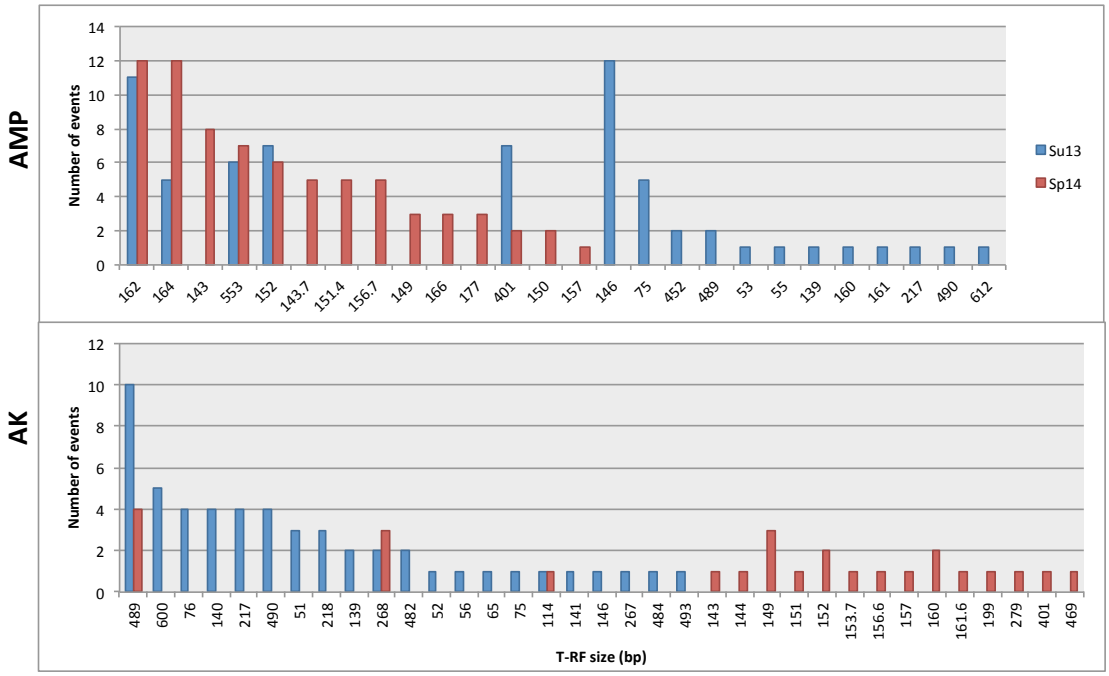


Figure 4.8 - Ranked event plot of OTUs observed in AMP and AK slurry in Su13 and Sp14.
More OTUs were shared seasonally in AMP slurry than in AK. For AMP Su13/Su14 n=12, AK Su13 n=10, and AK Sp14 n=4. Data was collected from consensus profiles generated from triplicate sample fingerprints.

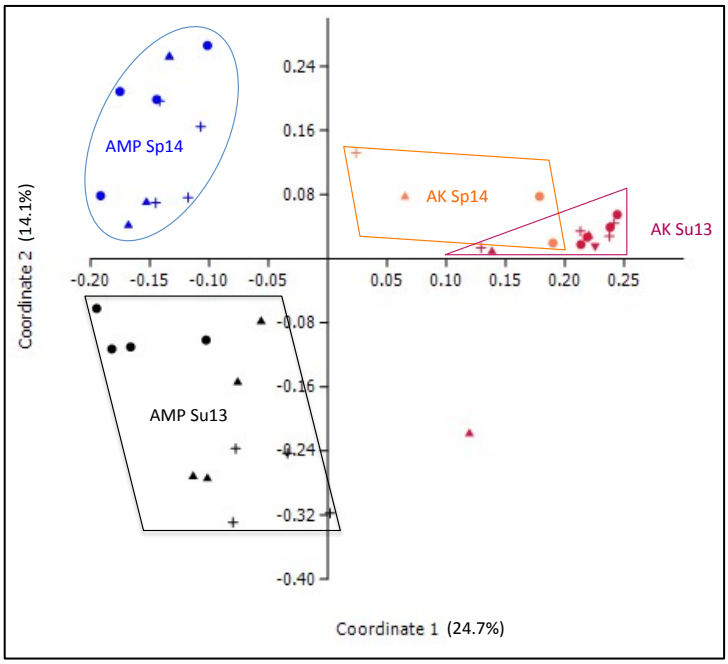


Figure 4.9 - Principal component analysis based on Jaccard distance of OTUs identified in AMP and AK slurry.
The AK samples grouped separately from the AMP samples. Su13 and Sp14 AK were similar and grouped along coordinate 2, while AMP Su13 was distinctly separated from AMP Sp14. Legend based on DNA extraction method: • DNeasy, ▲ Powersoil,+ DNeasy/PMA. Each point corresponds to the consensus T-RFLP profile for a given triplicate set.

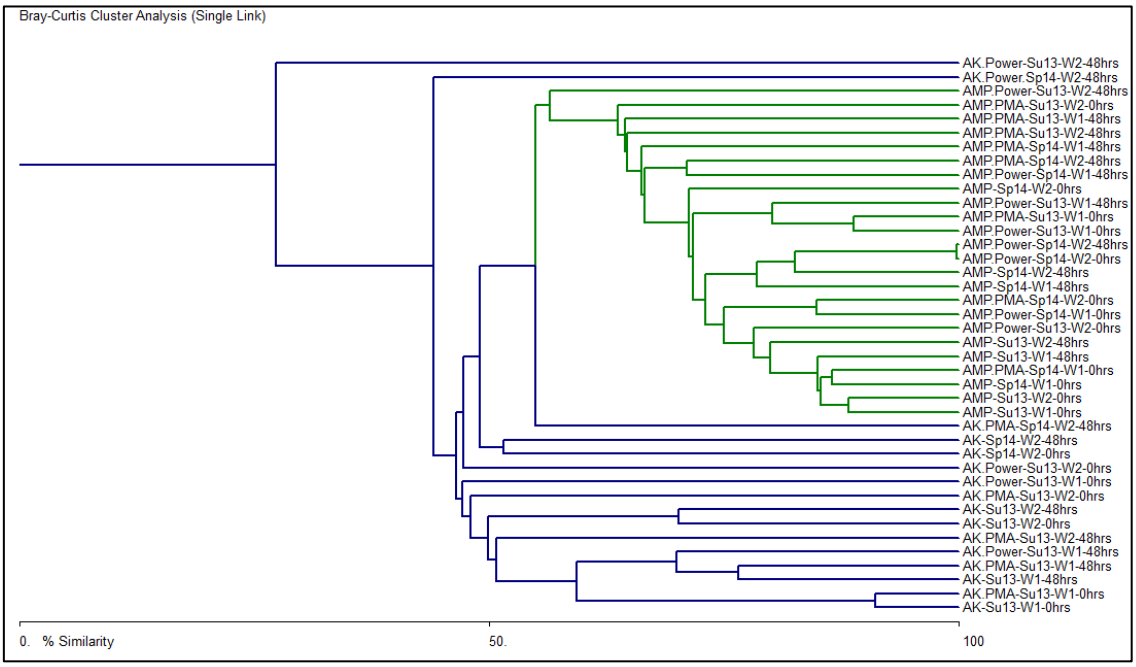
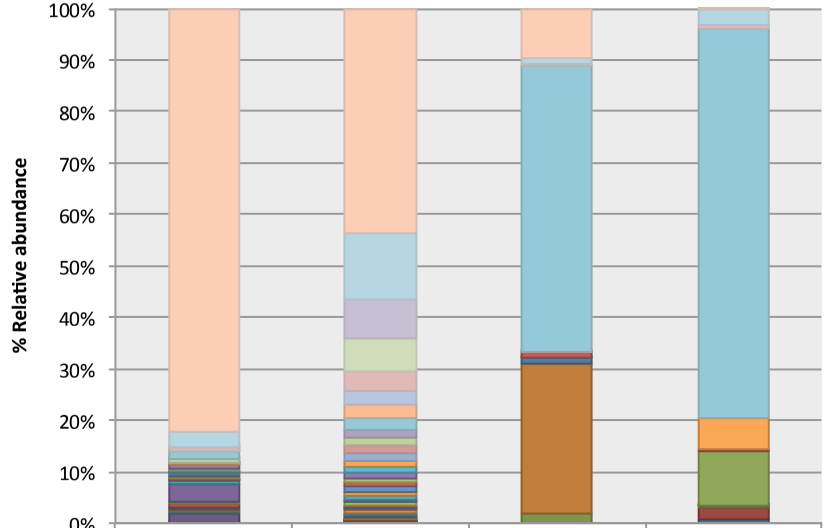


Figure 4.10 - Cluster analysis based on Bray-Curtis distance of OTUs identified in AMP and AK.

AMP samples (green) formed a distinct cluster from AK samples (blue). The species matrix was transformed by double-square root to diminish the influence of strongly dominant species in the samples.

A global overview of the sequencing data revealed that the microbial population in AMP and AK slurries were significantly different (R=0.72, $P < 0.001$ analysis of similarity [ANOSIM]). The Kruskal Wallis test based on Bonferroni-corrected p-values ($P < 0.05$) was performed to identify the taxa that were found to differ significantly in abundance between the slurries and seasons. AK slurry was dominated by *Pseudomonas* spp. while *Bacillus* spp. predominated in AMP (Figure 4.11). The high prevalence of *Bacillus* spp. was surprising and its poor recovery in culture (8-15% of the colonies, Figure 4.7) signified that the media and culture conditions were inadequate. The organisms in significantly higher abundance in AMP communities were *B. halodurans*, *B. cohnii*, *Novosphingobium sp.*, unclassified *Bacilli*, *Flavobacterium sp., Lysobacter taiwanensis* and *Nesterenkonia sp.* ($P < 0.05$) (Figure 4.11). The main taxa distinguishing AK populations from AMP belonged to the *Pseudomonas* genus, principally *P. mendocina* ($P < 0.05$) (Figure 4.11). Principal coordinates analyses of the sequence data were performed based on weighted UniFrac distances, taking into account the relative abundance of OTUs. The samples formed distinct groups according to AMP or AK in each season (Figure 4.12).



	AK Su13	AK Sp14	AMP Su13	AMP Sp14
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	82%	44%	10%	0%
Low abundant species comprising <1% of population	3%	13%	1%	3%
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other;Other	0%	8%	0%	0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Natranaerobiales;f__Anaerobrancaceae;g__Anaerobranca	0%	6%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__	1%	4%	0%	1%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other	0%	3%	0%	0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Intrasporangiaceae;g__Janibacter	0%	3%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	2%	2%	56%	75%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__;g__	0%	2%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus	1%	2%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__	0%	1%	0%	0%

Figure 4.11 continues on the next page.

k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Chryseobacterium	0%	1%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other	0%	1%	0%	6%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Hydrogenophaga	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingobium	1%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;g__Ochrobactrum	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Blastomonas	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales;f__Methylophilaceae;g__Methylotenera	0%	1%	0%	0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	0%	1%	0%	0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Paracoccus	1%	1%	0%	0%
k__Bacteria;Other;Other;Other;Other;Other	0%	1%	1%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0%	1%	0%	0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Microbacterium	0%	1%	0%	0%
k__Bacteria;p__OD1;c__ZB2;o__;f__;g__	1%	0%	0%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other	4%	0%	0%	0%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	0%	0%	0%	11%
k__Bacteria;p__[Thermi];c__Deinococci;o__Deinococcales;f__Deinococcaceae;g__Deinococcus	1%	0%	0%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0%	0%	1%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium	0%	0%	29%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Thermomonas	1%	0%	0%	0%
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	2%	0%	0%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Lysobacter	0%	0%	2%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__	0%	0%	0%	2%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Nesterenkonia	0%	0%	0%	1%

Figure 4.11 - Relative abundance based on 16S rRNA sequencing in AK and AMP samples from Su13 and Sp14.
A sampling depth of 2,200 sequences/sample was used to incorporate the greatest number of samples in the analysis. For AK n=126 and AMP n=132.

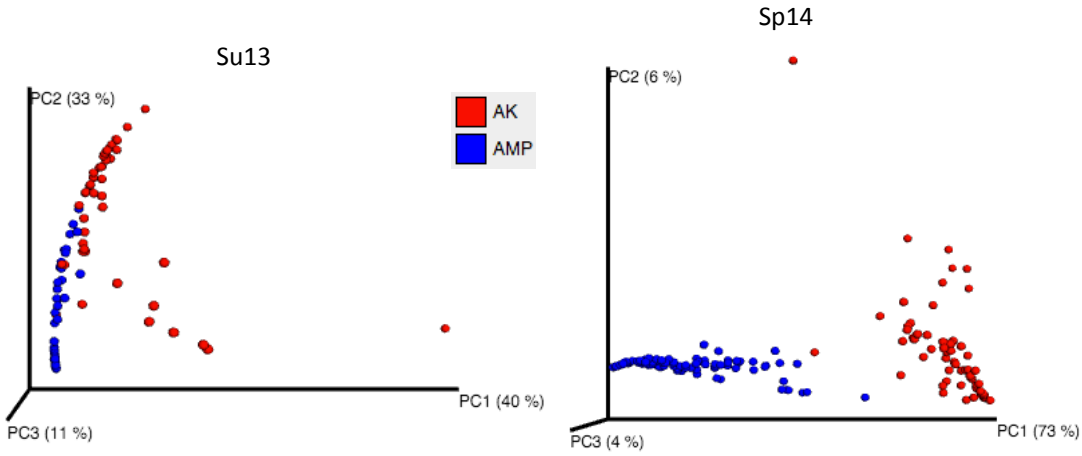


Figure 4.12 - Weighted UniFrac principal coordinate analyses plots illustrating the clustering of AK and AMP samples.
At a sampling depth of 2,000 sequences per sample in Su13 AK n=69, AMP n=92; in Sp14 AK n=72, AMP n=71.

Both slurries exhibited changes in diversity from Su13 to Sp14. In AMP *Novosphingobium* sp., *Pseudomonas* spp., and *P. mendocina* represented 39% of the total population in Su13 but were not detected in Sp14. Instead, Sp14 displayed a significantly higher proportion of unclassified *Bacillus* spp., *B. clarkii, B. halodurans*, *Flavobacterium* sp., and *Nesterenkonia* sp. ($P < 0.05$). In AK Su13 slurry 82% of the population belonged to *Pseudomonas* genus, including *P. pseudoalcaligenes* and *P. mendocina*. The decreased abundance of this genus in Sp14 and increased detection of *Bacillus* sp., *Anaerobranca* sp., *M. populi*, and *B. cohnii* helped differentiate the populations from the Su13 community ($P < 0.05$) (Figure 4.11).

Based on the T-RFs determined for the species isolates, the main organisms observed in AMP cultures were detectable within the community profiles. The 162 bp OTU (*B. halodurans*) and 146 bp OTU (*Novosphingobium* sp.) were observed the most frequently in Su13 and the T-RFs were recovered in 92% and 100% of all samples, respectively (Figure 4.8). The fragment of 162 bp was the most abundant at the first sample collection time point (0 hours, Week 1) and accounted for 60-80% of the population (Figure 4.13). This correlated with the MiSeq data identifying *B. halodurans* as 68-88% of the population (Figure 4.15). It is possible that the 162 bp OTU could comprise multiple taxa. Three OTUs were detected among the sequences classified as *B. halodurans*. In Su13 AMP, 51 OTUs were identified in the community but their classification was limited to a few organisms. Five OTUs were assigned to the genus *Novosphingobium*, three to *B. cohnii*, six to *Lysobacter taiwanensis*, 14 to *P. mendocina*, and 8 to the genus *Pseudomonas* (Figure 4.15).

As discussed previously this could be due to 16S rRNA variability in copy numbers, it could represent gene sequences from distinct strains, or it could be the result of insertion or deletion errors in PCR amplification.

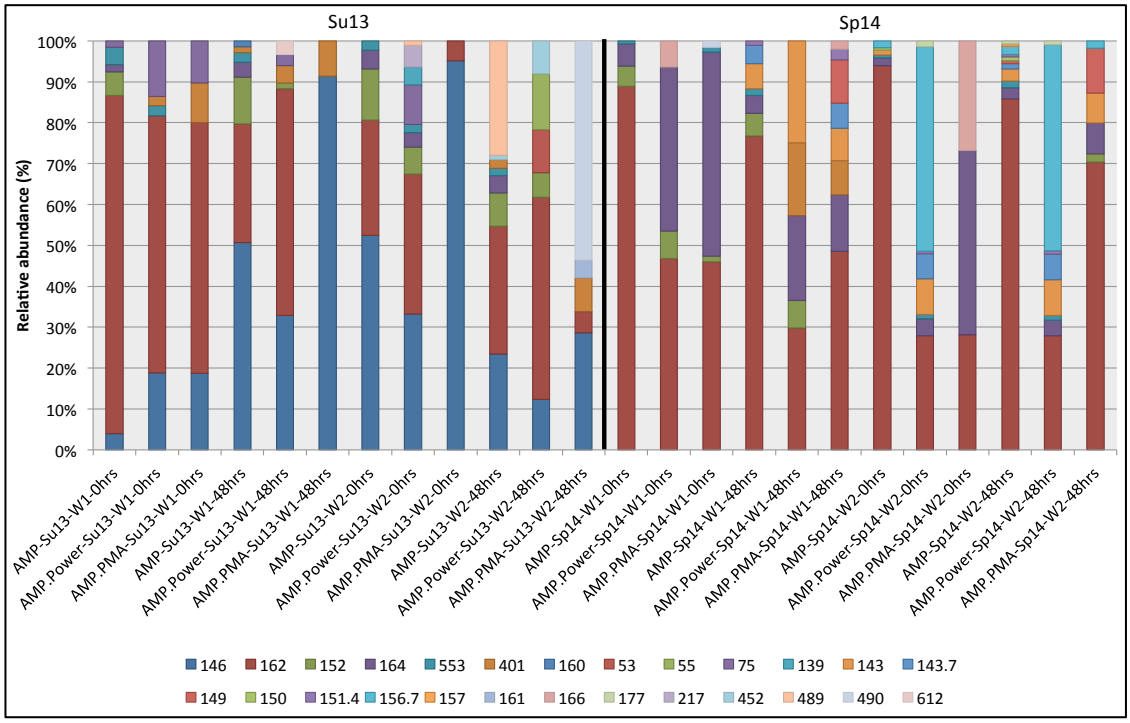


Figure 4.13 - Relative abundance of T-RFs observed in Su13 and Sp14 AMP samples.
Su13 consensus profiles were dominated by the OTUs 162 bp, 146 bp, and 152 bp. In Sp14 the most abundant OTUs in the consensus fingerprint were 162 bp, 164 bp, 156-157 bp, and 152 bp. Nomenclature: Slurry product-DNA extraction method-Week-time point. Unlabelled=Indirect extraction method; Power=direct method using the Powersoil kit; PMA=Indirect isolation of cells, PMA treatment followed by DNA extraction.

The contaminating population in AK slurry has been characterized by others (Di Maiuta, 2010; Schwarzentruber, 2003) and was still found to be dominated by the *Pseudomonas* genus. Only three OTUs were present in both seasons: 489 bp, 268 bp and 114 bp (Figure 4.8). 17 OTUs were found specifically in Su13 and 21 were detected in Sp14. Di Maiuta (2010) reported that T-RFs from 488-496 bp corresponded to the *Pseudomonas* genus. The fragments 489-490 bp were the most frequently observed in samples from both seasons (Figure 4.8). Together they accounted for 80-98% of T-RFs in the AK community in Su13 (Figure 4.14) and 82% of the sequences (Figure 4.11). Other OTUs that appeared in multiple profiles in Su13 included 600 bp, 76 bp, 140 bp, 217 bp, 490 bp, 51 bp, and 218 bp.

Higher species diversity was observed in Sp14 samples (Figure 4.11), most likely influenced by the low microbial load and concentration of DNA. *Pseudomonas* sp. was still dominant, but the relative abundance of *Pseudomonas* sp. template

decreased. PCR bias would be reduced facilitating the amplification and detection of gDNA from more taxa. Another explanation for the higher diversity was the presence of many low abundant taxa. Many singleton T-RFs were detected in both seasons in AK and this helped to inflate diversity. Unfortunately the bioburden level was too low in Sp14 AK, not enough template DNA was available, and the amplification yield was poor. As a result, most of the T-RFLP profiles prepared did not achieve a high enough total fluorescence to be included in analysis. The data from eight sample profiles were discarded, including all those from the PMA gDNA extract group, but four replicate sets were retained (Figure 4.14). The low template issue had less of an impact on sequencing. The multiple amplification steps during library preparation delivered enough PCR product that a sufficient sequencing depth could be achieved.

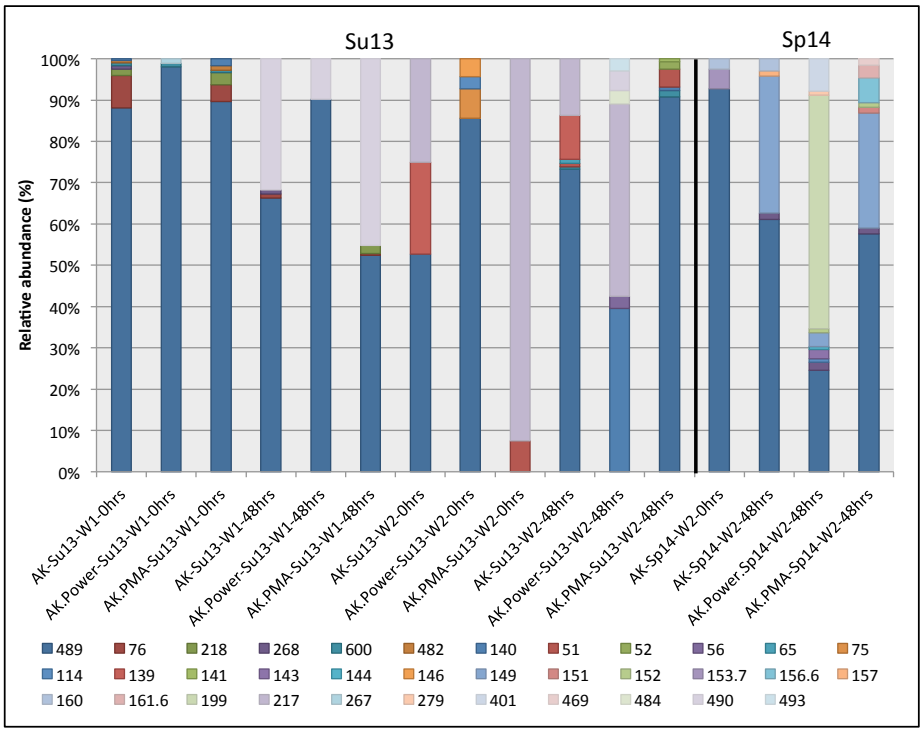
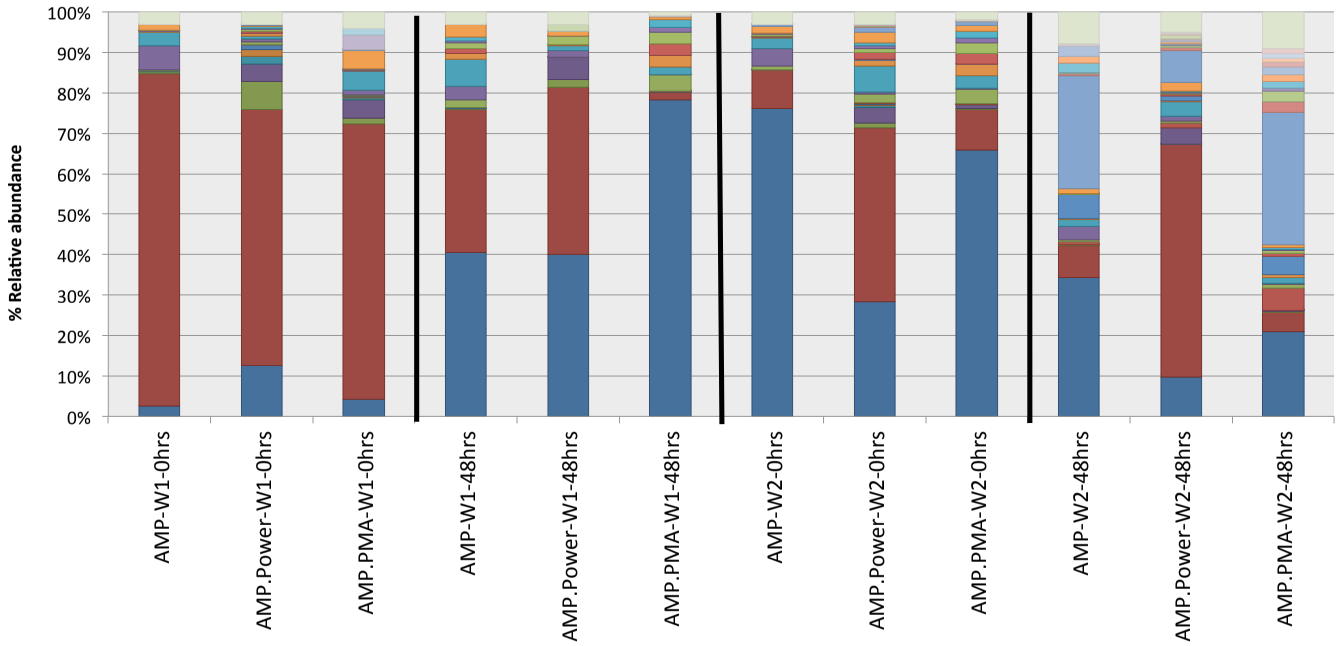


Figure 4.14 - Relative abundance of T-RFs observed in Su13 and Sp14 AK slurry.
The OTUs at 489-490 bp predominated in AK slurry during both seasons. Nomenclature: Slurry product-DNA extraction method-Week-time point. Unlabelled=Indirect extraction method; Power=direct method using the Powersoil kit; PMA=Indirect isolation of cells, PMA treatment followed by DNA extraction.

4.2.4.4.2 Influence of DNA extraction methods on community composition

B. halodurans was consistently observed in the AMP population but the data from the PMA-extracted samples suggested the viable population alternated in abundance. For example, after 48 hours of incubation the T-RF profiles and sequence data showed an increase in the abundance of the 146 bp OTU and *Novosphingobium* sp.. The gDNA sample from the indirect extraction method, herein referred to as "DNeasy", displayed a 2.6-fold increase in T-RF abundance (from 19% to 50%) (Figure 4.13) and 10-fold increase in the proportion of sequences attributed to *Novosphingobium* sp. (Figure 4.15). The direct DNA extract, to be denoted as "Power", exhibited a slight increase in the 146 bp OTU while the MiSeq data revealed *Novosphingobium* sp. abundance increased 3.4-fold after 48 hours (Figure 4.15). In both DNeasy and Power samples *B. halodurans* represented 35-41% of the population after incubation (Figure 4.13 and Figure 4.15). In contrast, the PMA-treated indirect extract, in which only DNA from viable cells was amplified, showed *Novosphingobium* sp. as more dominant representing 92% of the total community by T-RFLP (Figure 4.13) and several OTUs were combined to reflect 88% of the population by sequencing (Figure 4.15). The 162 bp OTU (*B. halodurans*) was not observed in the PMA sample suggesting a major shift in the population within 48 hours. It should be noted that the total RFUs for this sample were close to the 2,000 RFU threshold for exclusion from analysis, therefore the T-RF might not have been detected due to its lower abundance in the replicate sets of samples. *B. halodurans* was observed in 2% of the sequences (Figure 4.15) suggesting that its absence in the T-RFLP profile was due to low sensitivity of the method. This observation indicated that the *Novosphingobium* sp. fraction expanded rapidly during the incubation at 30°C, and this trend was undetected in the isolates from the other extraction methods.



k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__halodurans	0%	4%	5%	0%	5%	0%	0%	4%	1%	0%	4%	0%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__Lysobacter; s__taiwanensis	1%	7%	1%	0%	2%	0%	0%	1%	0%	0%	0%	0%
k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__halodurans	82%	63%	68%	35%	41%	2%	9%	43%	10%	8%	57%	5%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium; s__	3%	12%	4%	40%	40%	78%	76%	28%	66%	34%	10%	21%

Figure 4.15 continues on the next page.

k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	3%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	3%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	0%	0%	0%	0%	0%	0%	0%	1%	1%	28%	8%	33%
k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__cohnii	2%	0%	5%	3%	1%	1%	2%	3%	1%	1%	2%	1%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium	0%	0%	0%	1%	0%	2%	0%	1%	2%	0%	0%	0%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium	0%	0%	0%	1%	0%	1%	0%	1%	1%	0%	0%	0%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium	0%	0%	0%	1%	2%	3%	0%	1%	3%	0%	0%	1%
k__Bacteria	0%	1%	0%	1%	0%	3%	0%	2%	3%	0%	0%	1%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	1%	4%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae	0%	1%	0%	1%	0%	3%	0%	1%	3%	0%	0%	1%
k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__cohnii	3%	1%	5%	7%	1%	2%	3%	7%	3%	2%	4%	2%
k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__halodurans	6%	1%	1%	3%	2%	0%	4%	0%	0%	3%	1%	0%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium	0%	1%	0%	2%	0%	4%	1%	2%	4%	0%	1%	1%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	1%	5%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__Lysobacter; s__taiwanensis	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__Lysobacter; s__taiwanensis	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__Lysobacter; s__taiwanensis	0%	2%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%

Figure 4.15 continues on the next page.

OTUs representing < 1% of total sequences	3%	3%	4%	3%	3%	1%	3%	3%	2%	8%	5%	9%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__succinicans	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__mitochondria; g__; s__	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%
k__Bacteria; p__[Thermi]; c__Deinococci; o__Deinococcales; f__Deinococcaceae; g__Deinococcus; s__geothermalis	0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	1%	0%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	1%	2%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	2%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	2%
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__mendocina	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%

Figure 4.15 - Influence of DNA extraction method on Su13 AMP population diversity.
The sampling depth was 5,000 sequences/sample. Biological replicates produced nearly identical abundance estimates. For ease of visibility the data from one replicate was plotted. Nomenclature: Slurry product-DNA extraction method-Week-time point. Unlabelled=Indirect extraction method; Power=direct method using the Powersoil kit; PMA=Indirect isolation of cells, PMA treatment followed by DNA extraction.

Interestingly, the community analysis of the new sample received in Week 2 from the plant showed a similar trend. The PMA sample was predominated by the 146 bp OTU at 0 hours (Figure 4.13) and the MiSeq data exhibited four OTUs classified as *Novosphingobium* sp. at 76% of the population (Figure 4.15). Furthermore, another OTU at 3% was classified to the family *Sphingomonadaceae.* The DNeasy extract displayed a minimal change in the T-RF profile (Figure 4.13) conflicting with the sequencing results that indicated a population structure similar to the PMA sample. The Power extract showed little change to the community from the experimental sample at 48 hours (Week 1) according to abundance estimates by T-RFLP and sequencing (Figure 4.13, Figure 4.15). A shift in community composition was observed in Week 2 upon incubation of the slurry. Both the DNeasy and PMA populations contained T-RFs at 489 bp (*Pseudomonas* sp.) and 490 bp that were not detected previously (Figure 4.13). he sequence data confirmed the presence of the same *Pseudomonas* sp. in all three extracts (Figure 4.15). This inconsistency in the T-RF migration could have been due to T-RF drift (differences in fragment migration leading to improperly sized T-RFs) (Culman *et al.*, 2008). According to the community T-RFLP fingerprints, *Pseudomonas* sp. represented 55% of the viable population (PMA) compared to 28% of the total pool of indirectly extracted DNA, but was not recovered in the community isolated directly from slurry (Figure 4.13). The sequence data indicated *Pseudomonas* sp. contributed to 64%, 21%, and 62% of the DNeasy, Power, and PMA samples, respectively (Figure 4.15). The proportion of *Pseudomonas* sp. could have been lower in the Power sample because it represented a small fraction of the combined pool of viable and non-viable cells. It is possible that the contributing biomass of *B. halodurans* cells might have been so large that it masked any changes in community dynamics.

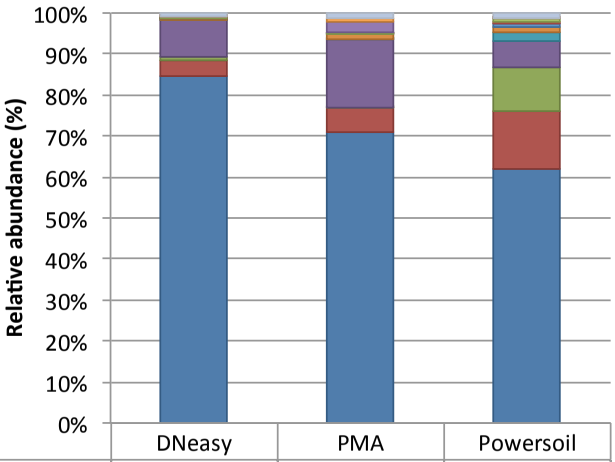
The final OTU detected by T-RFLP and sequencing was the fragment 152 bp corresponding to *B. cohnii*. The 152 bp OTU was observed in 58% of samples but not in PMA extracts (Figure 4.13). This lack of detection might have been due to its low abundance within PMA extracts of low concentrations. The T-RF comprised 6-12% of the total communities in DNeasy and Power samples (Figure 4.13). The level of detection was slightly increased by sequencing because *B. cohnii* was represented in 1-11% of the population across every sample (Figure 4.15). Another interesting finding was although *Brevundimonas* sp. was recovered in culture and 58% of samples in Su13 possessed the 401 bp T-RF, the fragment was observed in low abundance (2-10%) in the profiles (Figure 4.13). In addition, *Caulobacteraceae* genus was only detected in some samples representing <0.01% of the population

elucidated by sequencing. *Methylobacterium* sp. was also found in culture on PCA, yet the fragment 149 bp was not present in any sample profiles. It was also not observed in the analyzed sequences. The most likely explanation is it represented such a small fraction of the population that it was not efficiently amplified. However, it recovered successfully in culture because the conditions were more amenable to its growth than the *Bacillus* spp.. In the case of *Brevundimonas* sp., if there was initial bias against the low concentration of template in the first amplification reaction, this would have continued in the subsequent reactions. 16S rRNA sequences in high abundance would be preferentially amplified. Other commonly occurring OTUs in Su13 AMP produced fragment lengths of 75 bp, 164 bp, and 553 bp (Figure 4.8). The MiCA Virtual Digest tool can be used to predict T-RF length *in silico* under user defined amplification and digestion conditions (Shyu *et al.*, 2007). The fragment 165 bp was associated with *Bacillus spp.*, including *B. halodurans* C-125. Other strains of this organism would alternatively generate a T-RF at 554 bp. It is possible these OTUs corresponded to strains of *B. halodurans* that were not among the colony isolates selected for T-RFLP.

Overall DNA extraction methods did have a significant impact on community composition in AMP slurry in Su13 (R=0.14, $P < 0.001$, ANOSIM). Specific OTUs were observed in significantly higher abundance in samples extracted by a given method. In PMA samples *Pseudomonas* sp. and *Novosphingobium* sp. were more likely to be observed, while *B. halodurans* was expected to be present in lower abundance (G-Test, Bonferroni-corrected $P < 0.001$). In Sp14 AMP population structure was also influenced by the DNA extraction methods (R=0.39, $P < 0.001$, ANOSIM). In populations amplified from Power samples, one OTU identified as *B. halodurans* was less likely to be observed and OTUs classified to the *Bacillales* order (14%), *Bacillaceae* family (11%), and *B. cohnii* were more abundant than in the indirect DNA extracts (G-Test, Bonferroni-corrected $P < 0.001$) (Figure 4.16). Subsequent BLAST analysis of the OTU classified to *Bacillaceae* revealed a >97% sequence identity for *B. clarkii* and *B. polygoni*, recovered in culture, and a few other *Bacillus* sp.. Given the detection of this OTU in mainly the direct DNA extracts, it is possible this organism is closely associated with carbonate particles and is lost during the Histodenz cell separation. The aggressive bead-beating step in the Powersoil kit may be crucial for complete disassociation of these cells from the carbonate. The viable communities characterized from the PMA extracts had higher proportions of the OTUs *Flavobacterium* genus (16% compared to 9% in DNeasy extracts and 7% in Power samples) and *Methylobacteriaceae* family (3% versus 0% in DNeasy and Power isolates) (G-Test, Bonferroni-corrected $P < 0.001$) (Figure

4.16). DNeasy samples had the highest representation of *B. halodurans*, which highlights the bias imposed by the inclusion of DNA from dead cells (G-Test, Bonferroni-corrected $P < 0.001$) (Figure 4.16).

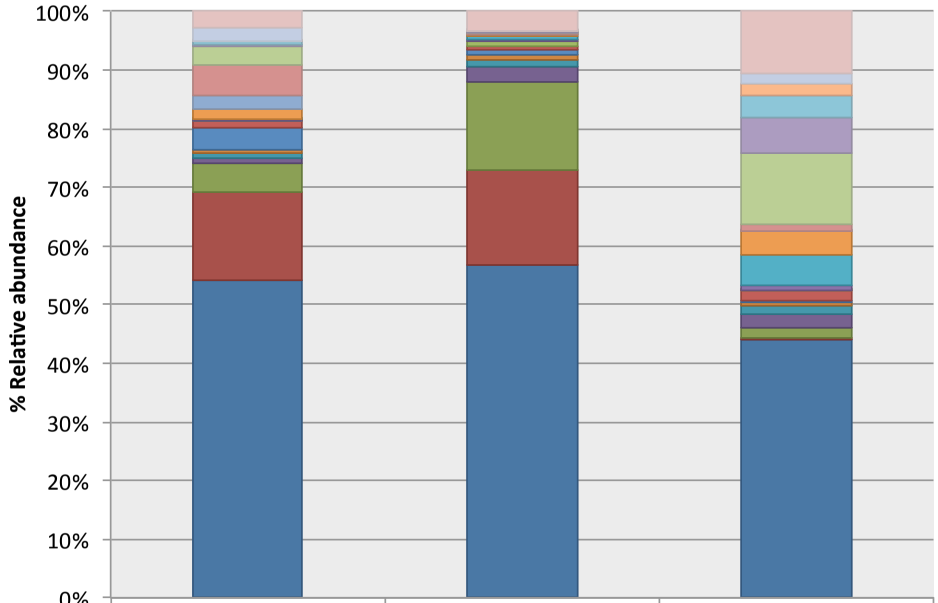
T-RFLP fingerprints of Sp14 AMP also contained the T-RFs 149 bp, 152 bp, and 162 bp that were observed and detected in 25%, 50% and 100% of samples, respectively (Figure 4.8). The 144 bp and 156-157bp OTU (*B. clarkii*) were observed in 75% and 42% of the profiles, respectively (Figure 4.8). The DNeasy fingerprints did not show much variation in composition from one time point to the next and *B. halodurans* remained the dominant T-RF (Figure 4.13). According to the corresponding sequencing data >90% of population belonged to the *Bacillales* order. There were slight changes in abundance of some fragments in Power extracts during the first week (T-RFs 143 bp, 152 bp, 164 bp, 401 bp) (Figure 4.13). Similar to the DNeasy samples most of the community were identified as *Bacillales* by sequencing. As mentioned earlier, during the fingerprinting analysis two peaks were occasionally observed together around 143.3 bp and 143.7 bp and were binned separately. The migration of the *Chryseomicrobium imtechense* T-RF was closer to 144, but it was thought that this second peak might have belonged to the same OTU. *In silico* digest using the MiCA tool (Shyu *et al.*, 2007) revealed that numerous *Bacillus spp.* would generate T-RFs in the range 143-145 bp. The sequencing data indicated that the *Planococcaceae* family, to which *Chryseomicrobium imtechense* belongs, only corresponded to 0.3% of all Sp14 sequences (1% in Power samples) whereas the *Bacillaceae* family represented >70% (Figure 4.16). Therefore, the main contributor of the T-RF 144 bp is more likely to be *Bacillaceae*. This is another example of the failure of the culture media. The plate cultures indicated *Chryseomicrobium imtechense* was dominant, but the conditions were not suitable for the recovery of alkaliphilic members of the *Bacillaceae* family. Finally, PMA Sp14 samples displayed the greatest shift in the population from 0 hours to 48 hours (Figure 4.13). Importantly, *Methylobacterium* sp. (149 bp) was detected with *B. clarkii*, 143 bp OTU, and *Brevundimonas* sp.. In the slurry analyzed in the second week these T-RFs were not observed at 0 hours but after incubation at 30°C for 48 hours they reappeared. Such shifts within the viable population were reliably discernible from analyzing the PMA extracts.



	DNeasy	PMA	Powersoil
OTUs representing < 1% of sequences	1%	1%	1%
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other;Other	0%	1%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__	0%	3%	0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other	0%	0%	1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0%	0%	1%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;Other	0%	0%	1%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Nesterenkonia	0%	1%	1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Lysobacter	0%	0%	2%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	9%	16%	7%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__	1%	0%	11%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other	4%	6%	14%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	84%	70%	61%

Figure 4.16 - Microbial community structure in Sp14 AMP according to DNA extraction method.
MiSeq sequencing results, sampling depth was 5,000 sequences per sample.

DNA extraction methods were also observed to have an impact on the recovery of community members in AK slurry. For instance, MiSeq sequencing results of Sp14 AK PMA slurry revealed *Methylobacteriaceae* family comprised 15% of the population compared to the 5% and 2% identified in DNeasy and Power communities, respectively (G-test, Bonferroni-corrected $P < 0.001$) (Figure 4.17). This information is particularly important for monitoring AK slurry given the contribution of *M. populi* and *M. extorquens* in formaldehyde resistance (AK resistance). The scale of the problem would be underestimated if one of the other DNA extraction methods were used alone. *Anaerobranca* genus, *Janibacter* genus (*Intrasporangiaceae)*, and *Sphingobium* genus were detected in higher abundance in Powersoil-extracted samples compared to the indirect DNA isolates (G-test, Bonferroni-corrected $P < 0.001$) (Figure 4.17). Although these organisms comprise 23% of the community in Power extracts, their relative abundance in the viable population is unknown. It is possible the cells are lost during the indirect extraction process, which would bias their representation in the total pool of community DNA. In contrast, an OTU classified to *Bacilli* was absent from Power samples but was 15-16% of the detected sequences in DNeasy and PMA (G-test, Bonferroni-corrected $P < 0.001$) (Figure 4.17). These specific differences were not readily detectable by T-RFLP (Figure 4.14) due to the lower sensitivity of the method.



	DNeasy	PMA	Powersoil
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__	4%	1%	0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae	1%	1%	1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	1%	1%	1%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]	1%	2%	2%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae	5%	15%	2%
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other	15%	16%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	54%	57%	44%

Figure 4.17 continues on the next page

OTUs representing < 1% of total sequences	3%	3%	11%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__	2%	0%	2%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales;f__Methylophilaceae	0%	0%	2%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae	0%	0%	4%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Intrasporangiaceae	0%	0%	6%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Natranaerobiales;f__Anaerobrancaceae	3%	0%	12%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other	5%	0%	1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae	2%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	2%	0%	4%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	0%	1%	5%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	0%	1%	1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Ellin329;f__	0%	1%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	1%	1%	2%

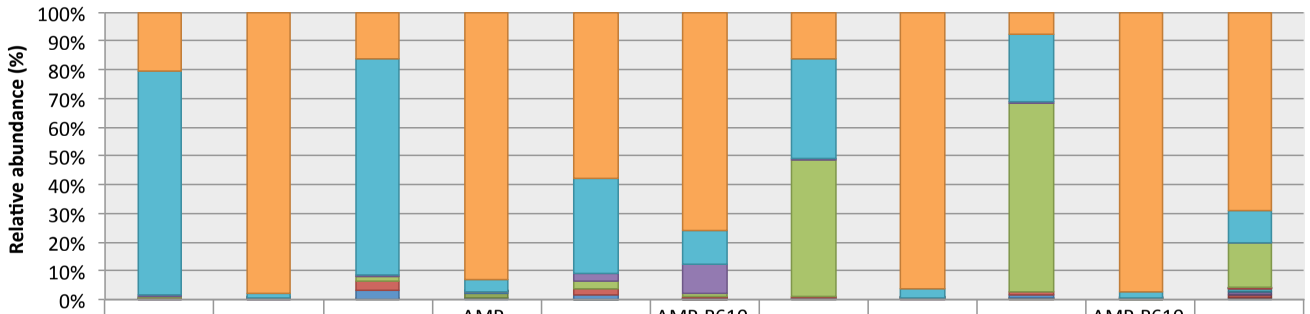
Figure 4.17 - Microbial community structure in Sp14 AK according to DNA extraction method.
MiSeq sequencing results, sampling depth was 8,000 sequences per sample.

4.2.4.4.3 The impact of biocide addition on community composition

Differences in community composition were evaluated between the control and R610 treatment groups. For example, in Su13 AMP samples *Novosphingobium* sp. and *P. mendocina* were detected in significantly higher abundance in the control group, while *B. halodurans* was more abundant in the R610 group (G-test, Bonferroni-corrected $P < 0.001$) (Figure 4.18). The data shows that both *Novosphingobium* sp. and *Pseudomonas* sp. were more susceptible to R610 than *B. halodurans*. At 0 hours *Novosphingobium* sp. dominated in the DNeasy and PMA extracts, while in the R610 treatment group *Bacillus* sp. corresponded to 93-98% of the population (Figure 4.18). After 48 hours of incubation, the community in the control group shifted to include *Pseudomonas* sp. (48% in the DNeasy isolate and 66% in the PMA extract) and 97-98% of the sequences recovered in the R610-treated samples were still from *B. halodurans* (Figure 4.18). This indicated that *Bacillus* sp. cells survived biocide treatment, which was also observed in culture (Table 4.1). The Power extracts showed a similar trend where a high abundance (76%) of the recovered sequences after R610 addition at 0 hours were from *B. halodurans*, however, the population also included 12% *Novosphingobium* sp. and 10% *Lysobacter* sp. (Figure 4.18). Since Power extracts include DNA from dead cells, the detection of *Novosphingobium* sp. was expected. The increased recovery of *Lysobacter* sp. sequences in the direct DNA isolates could indicate that a proportion of the cells may have been lost during the Histodenz cell isolation step (due to close association with the carbonate) resulting in the underrepresentation of this organism in the viable community (PMA extract). It could also suggest that a proportion of these cells may have evaded R610 biocidal activity.

AK slurry was also evaluated for changes to the population in response to R610 addition. In most DNeasy and Power isolates there was no significant difference observed between the control and R610 communities ($P > 0.05$). In Su13 PMA extracts the groups differed significantly in the abundance of specific *Pseudomonas* sp. OTUs, *B. halodurans* and other *Bacilli,* and *Methylobacteriaceae* family (G-test, Bonferroni-corrected $P < 0.001$) (Figure 4.19). *Methylobacterium* sp. in AK was shown to have survived the short-term exposure to R610 and regrow in culture so its detection was unsurprising. The presence of the class *Bacilli* was also explainable due to the high resistance of spore-forming organisms to biocides. However, this clearly indicated ineffective biocidal action of R610 since viable cells were detected. Sp14 PMA isolates also showed significantly higher abundance of *Bacilli* in the R610 group in addition to increased detection of low abundant taxa

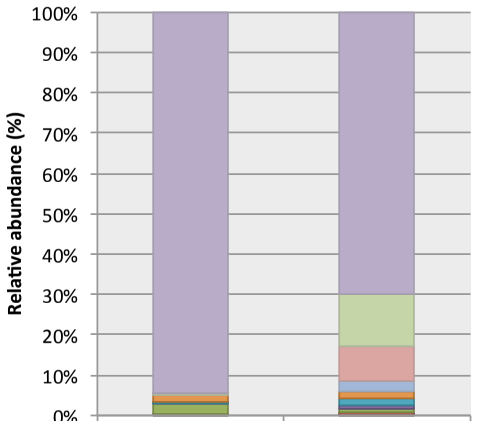
(each representing <1% of sequences within a sample) (G-test, Bonferroni-corrected $P < 0.001$) (data not shown). As mentioned previously samples with low microbial load and total DNA, such as most R610 extracts, showed an increase in the number of rare taxa since the proportion of dominant species decreases (in this instance *Pseudomonas* spp.). Although the identification of these low abundant organisms may not be of importance in the day-to-day microbial load management of slurry, this information contributes to our characterization of the communities.



	AMP-W2-0hrs	AMP.R610-W2-0hrs	AMP.PMA-W2-0hrs	AMP-R610.PMA-W2-0hrs	AMP.Power-W2-0hrs	AMP.R610.Power-W2-0hrs	AMP-W2-48hrs	AMP.R610-W2-48hrs	AMP.PMA-W2-48hrs	AMP.R610.PMA-W2-48hrs	AMP.Power-W2-48hrs
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	20%	98%	16%	93%	58%	76%	16%	96%	8%	97%	69%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium	78%	1%	75%	4%	33%	12%	35%	3%	23%	2%	11%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Lysobacter	0%	0%	0%	0%	3%	10%	0%	0%	0%	0%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	1%	0%	2%	0%	3%	1%	48%	0%	66%	0%	15%
k__Bacteria;Other;Other;Other;Other;Other	0%	0%	3%	0%	2%	1%	0%	1%	1%	0%	1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0%	0%	3%	0%	2%	0%	0%	0%	1%	0%	0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%
k__Bacteria;p__[Thermi];c__Deinococci;o__Deinococcales;f__Deinococcaceae;g__Deinococcus	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Thermomonas	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	1%

Figure 4.18 - Impact of R610 treatment on relative abundance of taxa in Su13 AMP slurry.

After R610 addition *Bacillus* sp., specifically *B. halodurans*, was recovered in all extracts. Sampling depth was 5,000 sequences/sample. One sample was excluded due to an insufficient number of reads. Nomenclature: Slurry product-DNA extraction method-Week-time point. Unlabelled=Indirect extraction method; Power=direct method using the Powersoil kit; PMA=Indirect isolation of cells, PMA treatment followed by DNA extraction.



	Control	R610
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	95%	69%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	0%	13%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__	0%	8%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium	0%	2%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other	2%	2%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0%	1%
k__Bacteria;p__[Thermi];c__Deinococci;o__Deinococcales;f__Deinococcaceae;g__Deinococcus	0%	1%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	2%	1%
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	1%	1%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae;g__Rhodococcus	0%	0%

Figure 4.19 - Relative abundance of taxa in Control and R610 treatment groups in Su13 PMA extracted AK slurry.
After R610 addition, *Pseudomonas* sp. still predominated but *Methylobacteriaceae* family and *Bacillus* genus were also detected. Sampling depth was 8,000 sequences/sample.

4.3 Conclusions and perspectives

Standard TSA and PCA media supported the growth of *Pseudomonas* sp., the most dominant organism in AK slurry, but failed to recover *B. halodurans* in AMP slurry in high numbers. The most likely explanation for this is the inadequate choice of media. *B. halodurans* is known to grow well on Horikoshi minimal medium containing glycerol and glutamic acid at pH 9-10 (Horikoshi, 2011). Despite the fact that high TVCs are still recoverable on the tested media, the counts are of less abundant organisms in AMP such as *Chryseomicrobium imtechense* and *Brevundimonas* sp. (although *Novosphingobium* sp. represented a dominant taxa). Culture analysis of AMP must be performed on other media in order to increase the detection of key taxa. Moreover, to maximize the recovery of species in culture it is essential to extend incubation times beyond the current practice of 48 hours.

T-RFLP provided valuable insight into the most abundant OTUs and the profiles of the bacterial isolates helped in assigning T-RFs. Performing the sequencing alongside T-RFLP was useful because it provided more context to the fingerprint, provided more confidence in the results, and enabled the identification and classification of organisms to the genus or species level. The sequence data mostly validated the sample profiles but also enabled the detection of organisms not recovered in culture or identifiable by the MiCA virtual digest tool such as *Flavobacterium* sp*., Lysobacter* sp., *Nesterenkonia sp.* and *Anaerobranca* sp.. T-RFLP requires a higher concentration of starting template DNA. For those samples not expected to contain large amounts of template, such as R610 treated slurry treated with PMA, the samples did not achieve a high enough level of fluorescence and many had to be excluded from analysis. The multiple PCR steps during MiSeq library preparation facilitated the amplification of these lower template samples. Another concern in the use of T-RFLP is the lower level of detection of rare taxa, an issue not encountered during sequencing. Nonetheless, it still remains an affordable option and could be an invaluable tool for routine monitoring of slurry communities. The identification of specific species is not always necessary during monitoring and the simple detection of a shift in community composition could in itself be useful.

Differences in community structure and species abundance were observed between the different DNA extraction methods. PMA treatment on Histodenz-isolated cells provided the most valuable characterization of the community. In comparison to the untreated extracts (DNeasy) several taxa were underrepresented including *Methylobacteriaceae* family, *Flavobacterium* sp. and changes in

abundance from one time point or week to the next were more perceptible. Significant shifts were observed within 48 hours of incubation, which was unexpected, demonstrating that the population was more dynamic than previously envisioned. This knowledge should serve as a caution to those characterizing communities from just one sample. It was previously known that the community composition alters throughout the seasons, but it was not known that changes could be perceived within days. This provides further indication that the population in AMP is not dormant. (It was once believed that the high alkalinity kept the cells in a stressed and inactive state (personal communications with N. Di Maiuta)). Furthermore, this reinforces the importance of collecting numerous samples from a given tank more regularly. A single sample provides a "snapshot" of the community and cannot deliver insight into the response and adaptation of the population to secondary biocide treatment. The amplification of products from the PMA samples indicates the survival of cells after R610 treatment. Although *Methylobacterium* sp. and *B. cohnii* were observed in culture in AK and AMP after R610 addition, respectively, the PMA extracts exposed the presence of more diverse surviving populations in slurries. This provides an explanation for the seemingly rapid regrowth and recovery of microbial loads after biocide dosing at the plants.

There are still benefits to performing the direct DNA extraction of samples using the Powersoil kit. Specific taxa belonging to the *Bacillales* order, *Anaerobrancaceae* family, *Intrasporangiaceae* family, and *Lysobacter* genus were recovered in higher abundance in Power community extracts. Therefore, to achieve the most accurate characterization of slurry populations it is necessary to apply both a direct DNA extraction method and an indirect DNA extraction of PMA-treated cells.

Chapter 5

Conclusions

Conclusions

Microbial monitoring in industry is necessary to ensure product safety for use and consumption and protect consumers, to prevent biofouling of surfaces, to maintain product characteristics and uphold quality, and to conserve a good company and brand image. Problems related to microbial contamination can have a devastating effect on business operations and put consumer health at risk. Classical culture methods have been the gold standard for quantifying bacterial load levels and detecting pathogens. They are technically simple, easy to interpret, affordable (in relation to some RMMs), and can be reliably used for the detection of specific organisms. Some of the limitations of these methods are the long time to result, they can be laborious, and the basic tools for enumerating bacteria such as dip slides are inaccurate. GMP and HACCP approaches are widely applied, even outside the food industry, to monitor and control products in-process rather than relying on finished product testing of bioburden. RMMs can provide increased sensitivity and specificity and near real-time results. Microbial problems can be detected earlier and corrective action can be applied sooner providing several financial benefits to business operators.

A wide-range of RMMs exist on the market but their uptake and application in industry has been slow. Some industries like pharmaceuticals and food are heavily regulated and must comply with regional legislation and guidelines. RMMs must be validated and have demonstrated equivalence to reference methods. This typically requires significant administrative coordination and input but enough RMMs have been through the validation process with the AOAC or ISO and later achieved approval that perhaps more interest in RMMs will develop. Investigating these alternative methods can be a daunting task. As discussed in Section 2.3.3.3 there are numerous and diverse technologies available and as a business operator the decision-making process can be complex. Each method has its advantages and disadvantages and some may be more suitable for use in some products or industries than others.

The technology must be fit for purpose; this can be ensured by careful consideration of a number of factors. First, managers must consider the stakeholders, including regulators, direct customers, and customers of the finished products. For instance, there may be strict product specifications such as detection limits or bioburden levels that must be met. Ideally this switch to an alternative method would not produce a perceptible change in the microbial quality of the product compared to the reference method. Second, it is important to reflect on the purpose of the test. Methods developed for verification and monitoring purposes

may have different requirements to those used in directing control activities or alerting QA managers to a problem. The latter two scenarios would benefit most from real-time detection systems while the former can handle a more extended time to result. Using Omya and its management of calcium carbonate slurries as an example, dip slides remain the preferred method for routine monitoring at the plants. Although the RMM CFII possesses the capability to identify problems in real-time and direct the timing of biocide addition, the managers were not accustomed to rapidly and proactively responding to data. Third, RMMs may not be suitable for use or perform equally well in all scenarios. For instance, the presence of interfering compounds within products can affect assay performance. This is an issue that has plagued ATP testing, as mentioned previously. Fourth, from a management perspective the cost per test and the time to result are important factors affecting operating costs. On the one hand, RMMs tend to be more expensive requiring large initial investments to purchase equipment, and costs to cover maintenance and training. On the other hand, many RMMs are semi or fully automated freeing up staff resources. In addition, rapid receipt of results allows for the early release of product to the market and for the appropriate corrective action to be applied in time to recover the product. Finally, business managers must understand the performance characteristics that are required of the technology (accuracy, detection limit etc.) and the operational requirements of the company. Referring again to the Omya and CFII case study, the main issues arose from the disagreement between these two factors. CFII is characterized by its high sensitivity and accuracy and Omya's Microbiology group envisioned its role in improving biocide management. However, the actual operators at the plants wanted a simpler monitoring method offering slightly increased accuracy compared to dip slides. The defining features of the CFII were not desired and the sophisticated but informative results were never exploited to their full potential. Because of this disconnect the technology struggled to gain support at the plants.

Omya faces many challenges going forward but these are difficult to perceive when the current outlook is positive. The strategy of pH stabilization has reduced the occurrence of post-production corrective treatments that plagued biocide-preserved slurries. The product is stable, the physiochemical characteristics are maintained, and high microbial loads are supported without adversely affecting product quality. Bioburden specifications are based on the lower limits known to cause problems with viscosity and acidification in AK slurry (10^4 cfu·mL^{-1}). This threshold limit was maintained by dosing with biocide as required. Omya may argue that microbial load is no longer relevant owing to the superior dispersion and pH

stabilizing qualities of AMP, MEA and other ethanolamine compounds being evaluated. However, whether it is apparent on the surface or not, the product has changed. It has been suspected by many at Omya that the residual biocide concentrations in biocide-preserved slurries were sufficiently high that they helped protect downstream processes at the customer site from heavy microbial contamination. This is one important aspect of the switch to pH stabilization; the customers no longer receive a protective benefit. A more significant way in which the product has changed is in the composition of the contaminating microbial community.

It was expected that the higher alkalinity would limit the growth of microorganisms in slurry. Investigations in the laboratory showed biocide-resistant communities in slurry displayed inhibited growth at defined concentrations of AMP (Di Maiuta and Schwarzentruber, 2013; Di Maiuta, 2010; Di Maiuta and Schwarzentruber, 2012). The method of pH stabilization was tested in a pilot-study and expanded to all large European plants. Over time the apparent growth inhibitory effects subsided as the population adapted to the higher pH. In the previous section common taxa in AMP slurry were identified and the community was dominated by alikaliphilic spore-forming *Bacillus* species, including *B. halodurans*, *B. clarkii*, *B. cohnii*. *Bacillus* spp. are major contributors to biofilm communities in paper machines (Kolari *et al.*, 2001; Desjardins and Beaulieu, 2003; Oppong *et al.*, 2000; Vaisanen *et al.*, 1991; Rättö *et al.*, 2005; Vaisanen *et al.*, 1998). Biofilms can cause corrosion of metal surfaces and machinery, they can cause discolouration of paper, and damage paper quality introducing holes (Kolari *et al.*, 2003). Moreover, *Bacillus* spp. heat-resistant spores can survive conditions during hot drying and cells can be carried into paper products destined for food-quality packaging (Vaisanen *et al.*, 1991; Kolari *et al.*, 2001). Paper manufacturing occurs in the open and supports ideal conditions for microbial growth. The density of contaminating cells can vary from 10^4-10^8 cfu·mL^{-1} (Rättö *et al.*, 2005). While previous versions of biocide-preserved product may have had a protective effect in manufacturing, AMP slurry is at risk of contributing to or exacerbating contamination problems. There are increased reports of fungal contamination in pH-stabilized slurry, as mentioned previously. Two fungal species were recovered in bacterial culture from Sp14 AMP and were identified as *Aspergillus sydowii* and *Penicillium chrysogenum* (data not shown). Meanwhile, the presence of bacterial spores has not been evaluated. This is of considerable concern and could impact upon Omya's desire to reach new markets and applications for calcium carbonate slurries.

There are some indications from plants that customers have begun asking for AMP product supplemented with biocide. In this study *B. cohnii* did initially recover after R610 treatment, but the biocide was still effective at killing a large proportion of the population. Given the high bioburden in AMP slurry, the presence of fungi, and high abundance of spore-forming bacterial strains, the combination treatment is a good preventative measure and should perhaps be adopted routinely. In order to determine the benefit or necessity of this cost-incurring measure it is necessary to evaluate its effectiveness on a smaller scale and by replicating industrial conditions.

The slurry community is more dynamic than previously realized and current methods for challenge testing do not accurately reflect conditions on an industrial scale. During this study slurry samples from the same product tank at the plant were received over two consecutive weeks. Slurry was aliquoted into replicate sample bottles and incubated for two days at 30°C. By analyzing amplified 16S rRNA genes from cells treated with the viability dye PMA, changes to the relative abundance of taxa were detectable even with short incubation times of 48 hours. It would have been interesting to perform the work over a longer period to observe the extent of variability in taxa representation in the population.

Challenge testing and MIC determination is typically carried out in artificially contaminated slurry. This can be achieved by inoculating sterile slurry with a single known bacterial isolate or a mixture of isolates in order to create a mock community. Heavily contaminated slurry can also be used to inoculate sterile slurry. The major drawback of these approaches is that it does not represent a natural community. Another criticism is that these studies are carried out in a closed system and do not accurately reflect the realities at the plant. Most product tanks are managed as a continuously-fed system for several weeks or months. Starting with fresh product from an industrial site, conditions can be replicated by occasionally mixing, removing product, and supplementing it with freshly delivered product. With this design it could be possible to setup a realistic slurry community and observe how diversity changes over time and responds to the selective pressure or antimicrobial being applied.

Such an investigation would greatly benefit from a multipronged approach, similar to what was described in the previous section: culturing under different media, bacterial load assessment by an RMM, and 16S rRNA gene analyses by T-RFLP and amplicon sequencing. Culturing can be a valuable tool, especially when the optimum culture conditions have been identified and the detection limits are known. Omya recently invested in a MALDI-TOF MS system that enables high-

throughput screening and rapid identification of colonies. In order to maximize the recovery of the slurry population by culture methods it will be necessary to exploit a wide range of media with varying nutrient and salt contents and ranges of pH, and by applying long incubation times and a range of growth temperatures. With MALDI-TOF the spectral patterns can change when organisms are in different phases of growth, are grown under different culture conditions, or when they are under stress. Species identification can be confirmed by 16S rRNA sequencing and these variations in spectra can be incorporated into the database. Although the method can be optimized, characterization of a population is still restricted to the culturable fraction, which may be an acceptable compromise depending on the information required from the samples. Accurate representations of population structure and diversity are invaluable in characterization studies and for understanding the impact of a given stress on the community. Once a better perception of the community and potential dynamics is established, it would be possible to reduce the number of methods applied. For instance, after associating different sized T-RFs with specific species it would be possible to rely on fingerprinting profiles alone to observe changes to the community. Although sequencing provides more accurate diversity estimates and species identification, it is still an expensive method and not suitable for single routine analysis.

In conclusion, the findings of this work demonstrated the necessity for due diligence in evaluating and applying new methods and technologies in industrial microbiology. It is important for businesses and users alike to understand inherent biases and to adopt an approach that does not leave one susceptible to oversights. Microbial communities are dynamic and always adapting and the methods used in monitoring and detection must keep pace.

Chapter 6

Materials and Methods

6.1 Materials and methods used in Chapter 3

6.1.1 Collecting information about Omya and its microbial management strategies

Much of the information regarding R&D work, sources of contamination, and managing customer complaints came from conversations with P. Schwarzentruber, N. Di Maiuta, J. Glaubitz, and S. Urwyler (past and present Heads of Microbiology or R&D). Information about biocide and preservative costs came from J. Glaubitz. F. Voorbraak and E. Brugman from NLMJ have also been invaluable in providing insight into the challenges of slurry management and in providing feedback on CFII. Quality and laboratory managers at NOME (J. Dyrhaug), NLMJ, ATGU (T.G. Sollund) and ITAV (L. Pappalardo) provided responses to a questionnaire covering the topics of:

- Volume of GCC produced
- Biocide and pH stabilizer usage
- Frequency of microbial-related complaints
- Areas for potential cost savings

The questions and responses can be found in Appendix II.

6.1.2 GCC slurries and preservatives applied

The water-based GCC slurries are prepared by wet-grinding of marble and limestone. The slurries analyzed during the pilot study were produced at the Omya Hustadmarmor AS plant in Norway and shipped to the Omya Netherlands BV tank farm (referred to as NLMJ). This site is a major distribution centre for paper manufacturers in Western Europe. The product specifications for the slurries being monitored are listed in Table 6.1.

Table 6.1 - CC slurries being tested

Trade name	Abbreviation	Solids (% w/w)	Particle size distribution	Preservative in use	
				AMP	AK
Hydrocarb 60	HC60	78	60% < 1 µm	✓	✓
Hydrocarb 75	HC75	78	60% < 2 µm	✓	✓
Covercarb 75	CC75	72	95% < 2 µm, 75% < 1 µm	✓	
Hydrocarb 90	HC90	78	90% < 2 µm, 63% < 1 µm	✓	✓
Hydrocarb 95	HC95	78	95% < 2 µm, 80% < 1 µm		✓

During the final stages of manufacturing the slurry is either dosed with OmyAK (The Dow Chemical Company) or with the pH stabilizer Corrguard-75 (AMP) (The Dow Chemical Company) (Table 6.2). In 2014 AK was replaced with Acticide BXL (Thor) but the formulation is the same. The biocide Rocima 610 (The Dow Chemical Company) (R610) is dosed when excessive microbial growth is detected during

storage. AK products are maintained around pH 9.0 and AMP products are stabilized at pH > 9.5.

Table 6.2 - Biocides and preservatives

Trade name	Supplier	Active compounds	Concentration (% w/w)	CAS No.
Corrguard-75	Dow	2-Amino-2-methyl-1-propanol	75.0	124-68-5
OmyAK / (Acticide BXL)	Dow / (Thor)	(Ethylenedioxy)dimethanol	85.0	3586-55-8
		2-Methyl-4-isothiazolin-3-one	0.25	2682-20-4
		5-Chloro-2-methyl-4-isothiazolin-3-one	0.75	26172-55-4
Rocima 610	Dow	Glutaraldehyde	22.5	111-30-8
		2-Methyl-4-isothiazolin-3-one	0.31	2682-20-4
		5-Chloro-2-methyl-4-isothiazolin-3-one	0.94	26172-55-4
		2-Bromo-2-nitro-1,3-propanediol	3.0	52-51-7

6.1.3 Microbial load assessment by culture methods

Slurry samples were collected from 13-15 storage tanks each day. E. Brugman opened sample ports on each tank for several minutes and allowed residual product in the line to flow out before collecting 50 mL of slurry. Aliquots were taken from each sample for dip slide testing, culturing on plate count agar (PCA) and tryptic soy agar (TSA).

6.1.3.1 Dip slide testing

Mikrocount® combi agar dip slides (Schülke&Mayr GmbH) were used to determine microbial load in slurry. One side of the plastic slide is coated with a bacterial growth agar (pH 7.1-7.4; triphenyltetrazolium chloride, peptone, meat extract, sodium succinate, NaCl, glycerol, agar) and the other is coated with Rose-bengal agar (pH 7.0-7.2; peptone, glucose, K_2HPO_4, $MgSO_4$, rose bengal, NaCl, Chloramphenicol, Gentamicin, agar) to detect the presence of fungi. Slurry was diluted 1:10 in 0.9% w/v NaCl. The sterile dip slide was dipped into the suspension for 1 minute, tapped gently to remove excess liquid, and returned to the storage container. Dip slides were incubated for 48 hours at 30°C and the total bacterial count per mL was determined by comparing the slide to the graphic in Figure 6.1. No replicates were performed since this is not the standard practice at the plants.

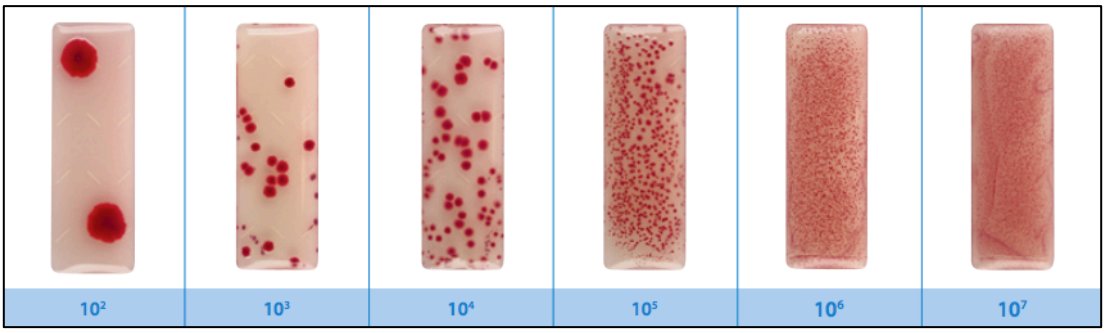


Figure 6.1 - Bacterial load estimation by dip slides.
Incubated slides are compared to the graphic to determine the total count (cfu·mL^{-1}). Image source: Schülke&Mayr GmbH (2015).

6.1.3.2 Plate culture testing

Microbial load was also assessed by culturing on plate count agar (PCA; 43558, bioMérieux SA) and tryptic soy agar (TSA; 43019, bioMérieux SA). 0.1 mL of undiluted or slurry diluted 1:10 (0.9% w/v NaCl) was spread on to culture plates and incubated at 30°C. The TVC was recorded after 48 hours.

6.1.4 Microbial load determination by CFII

The CFII system from CellFacts 2014 Ltd. (Coventry, UK) measures size and counts of microbial cells in a sample matrix. It combines electrical flow impedance and fluorescent staining to ascertain microbial load. The nucleic acid stain SYTO 62 (S-11344, Life Technologies) is used to detect all microbial cells within the sample. Viable cells are distinguished from non-viable particles by the uptake of 3,3-dipropylthiadicarbocyanine Iodide [diSC$_3$(5)] (CAS 53213-94-8, Life Technologies). diSc$_3$(5) is a membrane-permeant cation that only accumulates on hyperpolarized (viable) cellular membranes. Prior to each day's measurements the CFII was calibrated, cleaned, and programmed according to the manufacturer's instructions. Aliquots of the storage tank samples were also used for daily CFII analysis using the Histodenz and sedimentation sample preparation methods. Particle-size distribution profiles and total cell count (TCC) were generated using the CFII View software and algorithms.

6.1.4.1 Isolation of cells from slurry using Histodenz

Cells were isolated via density gradient centrifugation in 2 mL conical-bottomed microcentrifuge tubes. A 1.58 M solution of Histodenz (CAS. 66108-95-0, Sigma-Aldrich) was prepared in distilled water and sterilized by filtration. The solution was

stored at 4°C in 0.3 mL aliquots in the conical-bottomed tubes. Slurry was diluted 1:5 in 10mM Tris (pH 8.0, in 0.9% w/v NaCl) and mixed by vortexing for 3 minutes. 1 mL of diluted slurry was carefully pipetted into a tube containing Histodenz solution and centrifuged at 10,000 rcf for 6 minutes. The “Soft” key on the centrifuge was employed to slow the rate of acceleration and deceleration to maintain the density gradient. The clear upper aqueous layer containing the cells was removed to a sterile 1.5 mL tube and the carbonate pellet was discarded. The extract proceeded to fluorescent staining.

6.1.4.2 Cell staining of Histodenz-extracted cells

Working solutions of 100 µM $diSc_3(5)$ and 500 µM SYTO 62 were prepared in DMSO and stored at -20°C. 5 µL of 10mM EDTA (pH 8.0), 5 µL of $diSc_3(5)$ and 2 µL of SYTO 62 were added to each tube of HistoDenz-isolated cells. Tubes were mixed by vortexing and incubated at room temperature in the dark for 30 minutes. Cells were pelleted by centrifugation at 10,000 rcf for 5 minutes. The supernatant was discarded and the cells were resuspended in 1 mL of Resuspension buffer from CellFacts 2014 Ltd. (0.8% w/v polyethylene glycol in 0.9% w/v NaCl; adjusted to pH 7.5 with 0.2 M sodium phosphate buffer, pH 8.0). The sample information was entered into the CFII AutoSampler software and presented to the instrument for measurement and analysis.

6.1.4.3 CFII Sedimentation method

The fluorescent dye mixture was prepared from the working stocks at the beginning of each week: 150 µL $diSC_3(5)$ was mixed with 60 µL SYTO 62 and stored at 4°C. Slurry was diluted 1:10 in 10mM Tris (pH 8.0, in 0.9% w/v NaCl) in a 7 mL bijoux and mixed by vortexing for 3 minutes. The suspensions were placed on the CFII workstation in the specified rack. A 1.5 mL tube was prepared containing the diluted dye mixture and another containing 2% acetic acid. The volumes required were defined by the number of samples being analyzed (Table 6.3). Each column of the designated rack holds 13 samples. Reagent and sample tubes were placed in the positions as outlined in the user manual. Sample information was programmed into the software and the analysis was started. After 45-90 minutes of sedimentation (dependent on the slurry product) the instrument continued with the remainder of the sample preparation and proceeded with TVC measurements.

Table 6.3 - Volumes of diluted dye mixture and acetic acid required for setting up the CFII sedimentation assay

Number of samples	Dye tube		Acid tube
	Volume dye mixture (µL)	Volume ultrapure water (µL)	Volume 2% Acetic Acid (µL)
13	43	807	850
12	40	760	800
11	38	712	750
10	35	665	700
9	33	617	650
8	30	570	600
7	28	522	550
6	25	475	500
5	23	427	450
4	20	380	400
3	18	332	350
2	15	285	300
1	13	238	250

6.2 Materials and methods used in Chapter 4

6.2.1 Slurry samples

One HC90 AMP and one HC90 AK sample were collected at NLMJ and sent to the R&D Microbiology laboratory at the Omya Headquarters in Oftringen, Switzerland. Unlike the limited facilities at the plants, these laboratories are equipped for molecular biology work and large-scale culture experiments. Fresh samples were collected by E. Brugman from the same tanks in two consecutive weeks and used in the experiments described below and outlined in Figure 6.3. Experiments were carried out during late summer 2013 (Su13) and early spring 2014 (Sp14) to evaluate seasonal variations within the populations.

6.2.2 Experimental setup and design

Slurry was divided into a control group and a secondary treatment group (R610 group). Samples were prepared in triplicate as outlined in Figure 6.2. Both slurry products were setup in the same manner and the experiments for Weeks “1” and “2” during each season followed the same protocol.

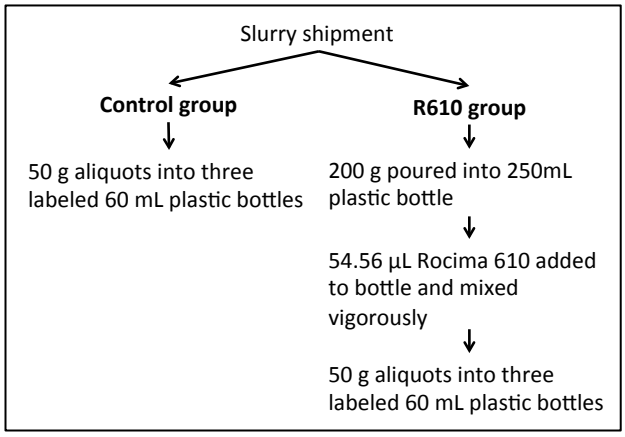


Figure 6.2- Preparation of triplicate biological replicates for slurry experiments.
50 g of slurry was poured into sterile plastic bottles. For the R610-treated group, R610 was added to a larger volume prior to aliquoting.

An overview of the sample processing steps and the methods applied is shown in Figure 6.3. The bottles were incubated at 30°C and aliquots were collected at 4 hours and 48 hours after R610 addition for culture plating, cell extraction and DNA isolation.

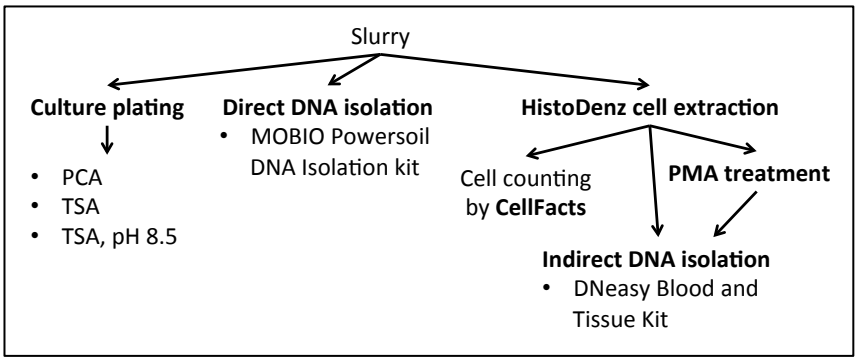


Figure 6.3 - Sample collection and workflow for downstream processing.
Samples were collected from each bottle using a 1 mL syringe.

6.2.3 Slurry challenge testing with R610

The HC90 slurry samples were already preserved with either AK or AMP. The biocide R610 (Table 6.2) was for the challenge tests. The MIC for R610 in HC90 products containing 78% (w/w) solids was previously determined to be 380 ppm per dry tonne of product (Di Maiuta, 2010). The density of the batch of R610 used in the experiments was 1.0867 kg/m^3 (according to MSDS specifications). The following equation was used to calculate the volume of R610 to add for secondary treatment of 200 g of slurry:

$$\frac{78\%\ solids \times 200\ g\ slurry \times 380\ ppm \times 1000}{10^6\ ppm \times 1.0867\ \ kg/m^3} = 54.56\ \mu L$$

6.2.4 Plate culture experiments

The microbial load was assessed by recording the TVC of 0.1 mL of slurry cultured on PCA, TSA, and TSA adjusted to pH 8.5 (Table 6.4). Two aliquots of slurry were collected from each sample bottle. One aliquot was spread directly onto the plates, the second was diluted 1:10 in 0.9% w/v NaCl before plating. Since slurry is highly viscous and opaque, the inclusion of the diluted set ensured maximal detection of colonies. Plates were incubated at 30°C and TVCs were determined after 2 days and 5 days. Photographs were taken to compare the density of growth on plates, colony morphology and species diversity. Colonies were randomly selected for 16S rRNA sequencing analysis from each sample plate set (discussed below). Plates were re-examined for further changes after 10 days. Statistical significance of bioburden levels and species abundance was analyzed by student's *t* test (two-tailed).

Table 6.4 - Composition of culture media

Media	pH	Composition (g/L)	
Plate count agar (PCA)	7.0	Pancreatic digest of casein	5.0
		Agar	15.0
		Yeast extract	2.5
		Dextrose	1.0
Tryptic soy agar (TSA)	7.3	Pancreatic digest of casein	15.0
		Papaic digest of soybean	5.0
TSA 8.5 (pH adjusted to 8.5 with NaOH)	8.5	Sodium chloride	5.0
		Agar	15.0

Final volume in 1 L distilled water. Media was sterilized by autoclaving at 121°C for 15 min.

6.2.5 Preparing cell extracts for CFII and PMA treatment

Cells were isolated using the Histodenz method described in 6.1.4.1. 1 mL of slurry was used to prepare three cell extracts for downstream analysis on the CFII, for DNA extraction, and for propidium monoazide (PMA) treatment.

6.2.6 CFII data analysis

Histodenz cell extracts were stained and measured on CFII as outlined in 6.1.4.2. Statistical analysis of mean TCC, mean cell size and mean fluorescence was performed using the two-tailed Student's *t* test (unpaired, equal variance).

6.2.7 Propidium monoazide (PMA) treatment of HistoDenz cell extracts

The cell extracts were pelleted by centrifugation at 10,000 rcf for 5 minutes. The supernatant was discarded and the cells were resuspended in 500 µL of Phosphate Buffered Saline (pH 7.4). The staining method was performed as described in the product information sheet from the manufacturer. Briefly, 1.25 µL of 20 mM PMA dye (CAS No. 40013, Biotium) was added to each tube, mixed gently, and incubated in the dark at room temperature for 5 minutes. A clear plastic tray was filled with ice and placed over a sheet of aluminium foil (to reflect the light). After incubation the tubes were laid horizontally on the ice and a 500 W halogen lamp was positioned 20 cm above the tray. The samples were exposed to light for 5 minutes and mixed occasionally by rocking the tray. The samples then proceeded to the indirect DNA extraction method described below.

Control samples were included during each PMA treatment. An extra HistoDenz cell extract was prepared and incubated at 95°C for 10 minutes to kill the cells. The cells were pelleted by centrifugation, resuspended in 500 µL of PBS and continued onto PMA staining. Prior to pelleting the cells for DNA extraction, 100 µL was reserved for culture plating to confirm the cells were killed.

6.2.8 Direct DNA extraction

The Powersoil® DNA Isolation Kit (Cat. 12888-100, MO-BIO) was previously shown to be suitable for isolating DNA from slurry (Di Maiuta, 2010). 750 µL of slurry was added to the Powersoil Bead tube provided and extraction proceeded according to the manufacturer's protocol using the vacuum manifold. The extracted DNA was stored at -20°C.

6.2.9 Indirect DNA extraction

The HistoDenz cell extracts were centrifuged for 5 minutes at 10,000 rcf. The supernatants were discarded and DNA was isolated from the cell pellets with the DNeasy® Blood and Tissue Kit (Cat. 69506, QIAGEN). The protocol was followed

as outlined in the manufacturer's handbook. DNA was eluted from the column using 100 µL Buffer AE and stored at -20°C.

6.2.10 Identification of bacterial isolates by 16S rRNA gene sequencing

For a given sample, a representative number of colonies were selected from each growth medium and dilution after 5 days incubation at 30°C. On media where colony colour and morphology was distinctive, five colonies were selected for each colony "type". Where the observed colonies were indistinguishable, ten colonies were randomly selected from the set of plate cultures. Colony lysates were prepared by transferring one colony to a 1.5 mL tube containing 50 µL of lysis buffer (10 mM Tris, pH 8.0; 0.5% TWEEN-20). The tubes were vortexed briefly and incubated and mixed for 10 minutes at 95°C in an Eppendorf ThermoMixer®. Lysates were centrifuged for 3 minutes at 10,000rcf and stored at -20°C.

Species identification was elucidated by amplification and sequencing of the V1-V6 region of the 16S rRNA gene using the EUB8m-f and EUB1088-r primers (see Table 6.5). The PCR reaction was setup in a final volume of 25 µL composing: 12.5 µL of HotStarTaq *Plus* Master mix (Cat. 203645, QIAGEN), 1 µL of each primer (10 µM working stock), 1.5 µL DMSO, 1 µL Bovine serum albumin (10 mg/mL), 6.5 µL sterile Nuclease-free water, and 1 µL of colony lysate. The cycling conditions included an initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 1 minute 15 seconds and a final elongation at 72°C for 10 minutes. The 16S rDNA sequences were compared to the GenBank database online using Basic Local Alignment Search Tool (BLAST, (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Madden, 2002). Species identification was also confirmed using the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) SeqMatch tool (Cole *et al.*, 2005). A phylogenetic tree of the 16S rRNA sequences was constructed in MEGA6 using the neighbour-joining method and evolutionary distances were calculated using the Maximum Composite Likelihood method (Hall, 2013).

Table 6.5 - Primer sequences used for 16S rRNA amplification and library preparation

Name	Sequence 5'→3'	Applications	Reference
EUB8m-f	AGAGTTTGATCMTGGCTCAG	16S rRNA sequencing, T-RFLP, MiSeq library preparation	(Weisburg *et al.*, 1991)
EUB338-f	ACTCCTACGGGAGGCAGC	16S rRNA sequencing, check primer MiSeq library	(Amann *et al.*, 1990)
EUB515-r	TTACCGCGGCKGCTGGC	16S rRNA MiSeq library preparation	(Baker *et al.*, 2003)
EUB1088-r	CTCGTTGCGGGACTTAACC	16S rRNA sequencing, T-RFLP	(Lee and Kemp, 1994)
8mf-rd1	TCGTCGGCAGCGTCAGATGT AGAGTTTGATCMTGGCTCAG	16S rRNA MiSeq library preparation	This study, modified from (Weisburg *et al.*, 1991; Illumina, 2013)
534r-rd2	GTCTCGTGGGCTCGGAGATG TATTACCGCGGCTGCTGGC	16S rRNA MiSeq sequencing library preparation	This study, modified from (Zongzhi Liu *et al.*, 2007; Illumina, 2013)
P5 Check	AATGATACGGCGACCACCGA GATCTACAC	Check primer MiSeq library	(Illumina, 2013)
P7 Check	CAAGCAGAAGACGGCATACG AGAT	Check primer MiSeq library	(Illumina, 2013)

6.2.11 16S rRNA T-RFLP

6.2.11.1 Generation of T-RFs

The universal bacterial primers EUB-8mf and EUB-1088r (Table 6.5) were used to target the V1-V6 region of the 16S rRNA gene. Triplicate 50 µL reactions were performed to yield 300-500 ng of amplified product for each gDNA extract. 10 µL of gDNA template was added to 40 µL of PCR Master mix composed of: 25 µL of HotStarTaq *Plus* Master mix, 1 µL of 6-Carboxyfluorescein (6-FAM) labelled EUB8m-f (10 µM), 1 µL EUB1088-r (10 µM), 1.5 µL DMSO, 1 µL BSA (10 mg/mL), 10.5 µL sterile Nuclease-free water. T-RF profiles were also prepared for 8 bacterial species isolated from slurry and identified through 16S rRNA sequencing: *Brevundimonas* sp., *Novosphingobium* sp., *B. cohnii, B. halodurans, B. clarkii, Ps. pseudoalcaligenes, Chryseomicrobium imtechense, M. extorquens.* 1 µL of lysate was diluted 1:10 in Nuclease free water and used as a template in the PCR Master mix described. Two distinct colony lysates were amplified per species. No template controls, where water was used instead of template DNA, were included on each PCR run. Amplification conditions consisted of an initial denaturation step at 95°C for 15 minutes followed by 33 cycles of 95°C for 45 seconds, 54°C for 45 seconds, 72°C for 2 minutes 30 seconds, and a final extension at 72°C for 30 minutes. Triplicates were pooled, purified using the QIAquick PCR purification kit (Cat.

28106, QIAGEN) and quantified by NanoDrop (Thermo Scientific). 300 ng of PCR product was digested with 3 units of *MspI* for 3 hours at 37°C and heat inactivated at 80°C for 20 minutes. The QIAquick Nucleotide Removal kit (Cat. 28306, QIAGEN) was used to purify the digested samples and to retain fragments greater than 40 bp in length. Samples were sent for fragment length analysis to Microsynth AG (Balgach, Swizterland): 2 µL of purified product (equivalent to 5-8 ng of RFs) was combined with 0.5 µL of the X-Rhodamine labelled size marker MapMarker 1000 (Cat. MM-1000-ROX, BioVentures) and 17.5 µL of highly-deionized formamide (Cat. 4311320, Life Technologies).

6.2.11.2 Analysis of T-RF Profiles

T-RF data were analyzed in Peak Scanner™ v2.0 (Life Technologies) using the default Local Southern method for size-calling. After visual inspection of electropherograms, a threshold limit of 50 fluorescence units was applied to distinguish background noise from 'true peaks'. The data was exported to MS Excel (Microsoft, USA) and the peaks below this limit were removed. T-RFs were also filtered by removing fragments less than 50 bp and greater than 1000 bp in length. Samples with a total peak height less than 2000 fluorescence units were excluded from analysis. Peak Scanner uses the nearest integer rounding method to assign fragment sizes into bins, where each integer is treated as a bin. The error in capillary electrophoresis in determining fragment sizes is ± 0.5 bp (Dunbar *et al.*, 2001); therefore, rapid manual inspection of bins was required to ensure that T-RFs differing by less than 0.5 bp in different profiles were assigned to the same bin. Samples were then normalized by calculating the relative abundance of T-RFs according to their peak height. Each raw peak height was divided by the total peak height of a sample. T-RFs contributing to less than 1% of the total were removed and the relativized peak height was recalculated. A derivative profile was created from the set of biological replicates by excluding irreproducible T-RFs (those that were observed in less than 100% of all replicate profiles) and by calculating the average peak height of each reproducible T-RF (Dunbar *et al.*, 2001).

The abundance data was imported into the BioDiversity Professional software (McAleece *et al.*, 1997) to create Bray-Curtis distance matrices and perform nearest neighbour cluster analysis. Principal Component Analysis (PCA) based on Jaccard distance were prepared using the Past analysis software (Hammer *et al.*, 2001). Analysis of variance (ANOVA) was used to determine the

statistical significance between differences in the means of the total number of T-RFs.

6.2.12 16S amplicon sequencing on Illumina MiSeq

6.2.12.1 16S rRNA library preparation

The V1-V3 region of 16S was amplified for 300 bp paired-end sequencing on the Illumina MiSeq. The library preparation workflow includes three PCR steps and is a modification of the 16S metagenomic sequencing library preparation method from Illumina Inc. (San Diego, CA, USA) (Illumina, 2013). The first PCR targets the 16S, the second PCR adds on the Illumina "Read1" (Rd1) and "Read2" (Rd2) sequences on the 5' and 3' ends of the 16S product (respectively) and the final PCR attaches the Illumina index identifiers "i5" and "i7" and the flow cell-binding sequences "P5" and "P7" to the 5' and 3' ends (Figure 6.4).

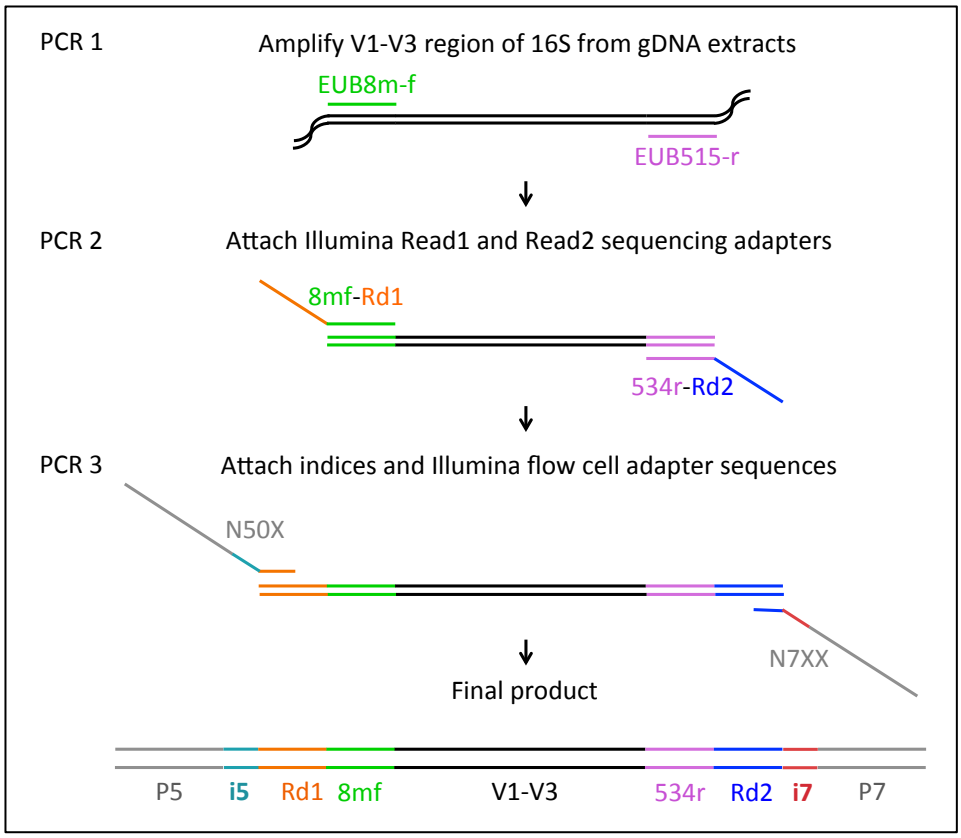


Figure 6.4 - MiSeq library preparation by three successive PCR reactions.
The V1-V3 region of 16S was amplified in PCR 1. PCR 2 used modified 16S primers with overhangs containing the Illumina "Read" sequences. In PCR 3 the Illumina index and adapter sequences are added to the product by use of overhang primers binding within the Read 1 or Read 2 sequences. This approach generated a final product in a single orientation with the P5 and i5 sequences at the 5' end and the i7 and P7 sequences at the 3' end.

Four libraries containing 89 samples each were prepared for four MiSeq runs. Each run contained one set of technical replicates (of a triplicate biological sample set on that plate). A mock community sample was included as an inter-run control. This was prepared by pooling the 16S rRNA PCR products of *Brevundimonas* sp., *Novosphingobium* sp., *B. cohnii, B. halodurans, B. clarkii, Ps. pseudoalcaligenes, Chryseomicrobium imtechense, M. extorquens*. Only one sample was prepared and processed alongside the other samples. All PCR reactions described below were carried out in a final volume of 50 µL containing 25 µL MyTaq™ Red Mix, 2x (Cat. BIO-25044, Bioline). Primer sequences for PCR 1 and 2 are listed in Table 6.5.

PCR 1 − 8 µL of extracted gDNA, 16 µL of NFW, 0.5 µL of EUB8m-f (10 µM), 0.5 µL of EUB515-r (10 µM) were added to the MyTaq Red Mix. The cycling parameters started with a denaturation step at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, and ended with a final extension step at 72°C for 10 minutes. The amplified products were checked by gel electrophoresis.

PCR 2 − PCR 1 products with good amplification were diluted 1:10 in NFW and reactions with weak or non-visible bands were left undiluted. 1 µL of this product was used as template DNA and added to the MyTaq Red Mix, 23 µL of NFW, 0.5 µL of 8mf-Rd1 (10 µM), 0.5 µL of 534r-Rd2 (10 µM). The cycling conditions were 98°C for 3 minutes, 20 cycles of 98°C for 30 seconds, 62.7°C for 30 seconds, 72°C for 1 minute 30 seconds, and a final extension at 72°C for 5 minutes. Gel electrophoresis was performed to check product size and yield. All reactions were purified according to the vacuum manifold protocol using the QIAquick PCR purification kit. Purified products were diluted 1:2 or 1:10 in NFW depending on the estimated yield by band intensity on the gel.

PCR 3 − Modifications of the N502-N508 and N701-N712 primers (Illumina, 2013) were used (Appendix III). The PCR was setup in a 96-well plate as described in the 16S library preparation protocol from Illumina. Since each N50X and N7XX primer has a unique 8 bp index sequence, each well contains amplified products with a distinct combination of 5' and 3' indexes to facilitate sample pooling and multiplex sequencing. 1 µL of the diluted purified PCR 2 product was added to 17 µL of NFW, 3.5 µL of forward primer N50X (2 µM), 3.5 µL of reverse primer N7XX (2 µM), and the MyTaq Red Mix. The cycling conditions were: 72°C for 3 minutes, 95°C for 30 seconds, 12 cycles of 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes. Product sizes were confirmed by running on a gel.

6.2.12.2 Product purification and normalization

Samples were purified with the Agencourt AMPure XP system (Cat. A63881, Beckman Coulter) according to the manufacturers instructions for the 96-well format procedure with the following modifications: $(Volume\ AMPure\ XP\ per\ reaction) = 0.65\times(Reaction\ volume)$; 80% Ethanol was used in the washes; products were eluted in 25 µL of EB buffer from QIAGEN (10 mM Tris-HCl, pH 8.5). Eluted products were normalized by the SequalPrep™ Normalization Plate Kit method (Cat. A10510-01, Life Technologies). The single mock community sample was divided across four SequalPrep plates. The kit estimates the yield per well to be 1-2 ng/µL. To verify normalization across the plate, eight wells were randomly selected per plate and quantified using the Qubit® dsDNA BR Assay Kit (Cat. Q32853, Life Technologies) and the Qubit® 2.0 Fluorometer (Life Technologies). Samples were pooled into a single tube by collecting 10 µL from each well. The estimated concentration of the pooled library was 2 nM. To achieve a final concentration of 4 nM for sequencing on MiSeq, 20 µL of the pooled library was centrifuged in vacuum concentrator for 20 minutes at room temperature. The final volume was adjusted to 10 µL with NFW. A 5% spike-in of the PhiX control was applied to each run.

6.2.12.3 Data analysis in QIIME

The raw data from each run was returned as four FASTQ files representing the forward and reverse sequences (Read1 and Read2) and the corresponding Index1 and Index2 reads. All steps were carried out in the QIIME 1.8.0 environment under the default parameters unless specified otherwise (Caporaso *et al.*, 2010). An overview of the workflow and scripts used is shown in Figure 6.5 and the specific input arguments are detailed in Appendix IV. The first few data preparation steps were carried out for each run individually. The two FASTQ files from the index reads were combined using the *combine_fastq_barcodes.py* script (available at: https://gist.github.com/walterst/6284164/revisions). The output file was used in the *join_paired_ends.py* script via the "*-b*" option. The *split_libraries_fastq.py* script was run at Phred quality scores (Q) ≥ Q30 and specifying a 16 bp combined index with the "*--barcode_type_16"* option. Sequences were aligned to the Greengenes core set (13_08 release, at 97% identity) (DeSantis *et al.*, 2006). Taxonomy was assigned using the RDP Classifier (Wang *et al.*, 2007) under the "*-m*" option in the script. OTUs with sequences representing <0.005% of the total sequences were filtered from the OTU table (Bokulich *et al.*, 2013). The *split_otu_table.py* and *filter_otus_from_otu_table.py* were used to separate samples into different

categories (i.e. product type, extraction method etc.) for ease of comparison and analysis. The majority of figures and statistics were generated using the script *core_diversity_analysis.py*.

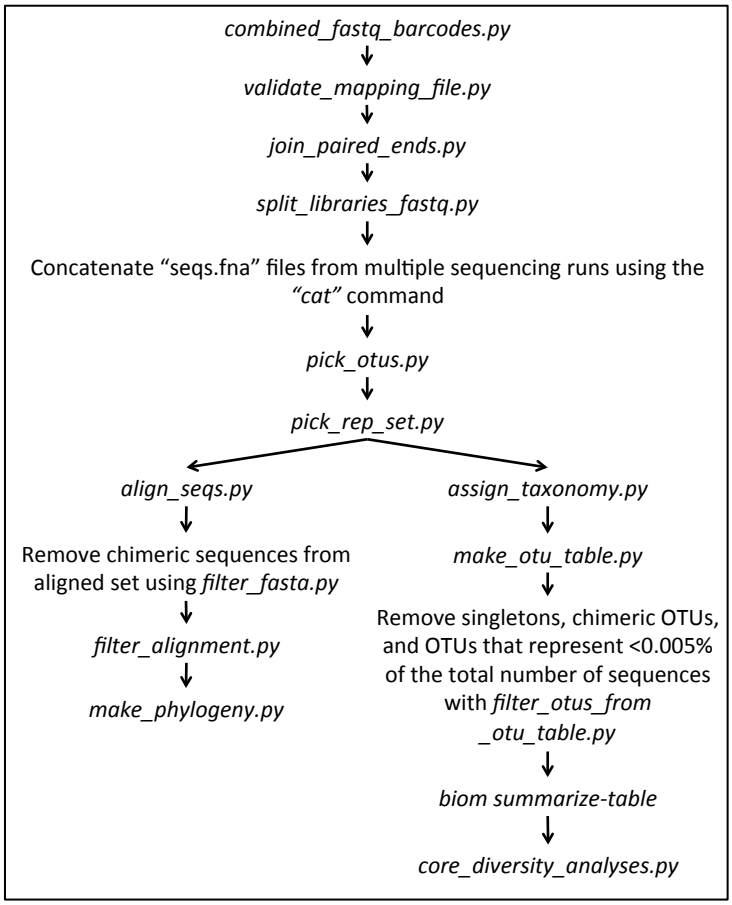


Figure 6.5 - Process flow of 16S rRNA sequence data analysis in QIIME 1.8.0. The default parameters were used unless specified otherwise.

References

Aino, K., Hirota, K., Matsuno, T., Morita, N., Nodasaka, Y., Fujiwara, T., Matsuyama, H., Yoshimune, K., Yumoto, I. (2008) Bacillus polygoni sp. nov., a moderately halophilic, non-motile obligate alkaliphile isolated from indigo balls. *International journal of systematic and evolutionary microbiology*. **58**(Pt 1), 120–4.

Alonso-Calleja, C., Guerrero-Ramos, E., Alonso-Hernando, A., Capita, R. (2015) Adaptation and cross-adaptation of Escherichia coli ATCC 12806 to several food-grade biocides. *Food Control*. **56**, 86–94.

Amann, R.I., Blinder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990) Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Applied and environmental microbiology*. **56**(6), 1919–25.

Amann, R.I., Ludwig, W., Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation . Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. *Microbiological reviews*. **59**(1), 143–169.

Angus_Chemical_Company (2015a) AMP Technical Data Sheet. *Angus Chemical Company website*, 1–2. [online]. Available from: http://www.angus.com/literature/downloaddoc?fileName=ANGUS_AMP_SalesSheet.pdf&contentType=Sales Sheet [Accessed July 15, 2015].

Angus_Chemical_Company (2015b) Corrguard-95 Technical Data Sheet. *Angus Chemical Company website*, 1–7. [online]. Available from: http://www.angus.com/literature/downloaddoc?fileName=ANGUS_CORRGUARD_95_TDS.pdf&contentType=Technical Data Sheet [Accessed July 10, 2015].

APCJ (2014) Going for growth with biocides. *Asia Pacific Coatings Journal*. **27**(3).

Arora, P.K., Chauhan, A., Pant, B., Korpole, S., Mayilraj, S., Jain, R.K. (2011) Chryseomicrobium imtechense gen. nov., sp. nov., a new member of the family Planococcaceae. *International journal of systematic and evolutionary microbiology*. **61**(Pt 8), 1859–64.

Atrih, A., Zollner, P., Allmaier, G., Williamson, M.P., Foster, S.J. (1998) Peptidoglycan structural dynamics during germination of Bacillus subtilis 168 endospores. *Journal of Bacteriology*. **180**, 4603–4612.

Azevedo, N.F., Bragança, S.M., Simões, L.C., Cerqueira, L., Almeida, C., Keevil, C.W., Vieira, M.J. (2012) Proposal for a method to estimate nutrient shock effects in bacteria. *BMC Research notes*. **5**(422), 2–7.

Bailey, A.M., Constantinidou, C., Ivens, A., Garvey, M.I., Webber, M.A., Coldham, N., Hobman, J.L., Wain, J., Woodward, M.J., Piddock, L.J. V (2009) Exposure of Escherichia coli and Salmonella enterica serovar typhimurium to triclosan induces a species-specific response, including drug detoxification. *Journal of antimicrobial chemotherapy*. **64**(5), 973–985.

Baker, G.C., Smith, J.J., Cowan, D.A. (2003) Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods*. **55**(3), 541–555.

BD (2015) INDUSTRIAL MICROBIOLOGY: BD FACSMicroCount™ System. [online]. Available from: http://www.bd.com/ds/im/microcount/index.asp [Accessed

August 20, 2015].

Bigger, J.W. (1944) Treatment of Staphylococcal Infections with Penicillin by Intermitent Sterilisation. *The Lancet*. **244**(6320), 497–500.

bioMerieux_SA (2015a) BacT/ALERT® 3D: Fully automated microbial detection system. [online]. Available from: http://www.biomerieux-industry.com/food/microbial-detection-bactalert [Accessed August 19, 2015].

bioMerieux_SA (2015b) CHEMUNEX® Rapid microbiology detection systems. [online]. Available from: http://www.biomerieux-industry.com/food/chemunex-flow-cytometry-analysers?dest=view [Accessed August 15, 2015].

bioMerieux_SA (2015c) VIDAS: Automated food pathogen detection. [online]. Available from: http://www.biomerieux-industry.com/food/vidas-systems [Accessed August 15, 2015].

Biswas, S., Rolain, J.M. (2013) Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *Journal of Microbiological Methods*. **92**(1), 14–24.

Blackwell, C.M., Scarlett, F.A., Turner, J.M. (1976) Ethanolamine catabolism by bacteria, including Escherichia coli. *Biochemical Society transactions*. **4**(3), 495–7.

Bogosian, G., Bourneuf, E. V. (2001) A matter of bacterial life and death. *EMBO reports*. **2**(9), 770–774.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G. (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature methods*. **10**(1), 57–9.

Bottari, B., Santarelli, M., Neviani, E. (2015) Determination of microbial load for different beverages and foodstuff by assessment of intracellular ATP. *Trends in Food Science & Technology*. **44**(1), 36–48.

Brown, M.R., Collier, P.J., Gilbert, P. (1990) Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. *Antimicrobial agents and chemotherapy*. **34**(9), 1623–1628.

Brown, M.R., Gilbert, P. (1993) Sensitivity of biofilms to antimicrobial agents. *The Journal of Applied Bacteriology Symposium Supplement*. **74**(Supplement S22), 87S–97S.

Brugger, S.D., Frei, L., Frey, P.M., Aebi, S., Mühlemann, K., Hilty, M. (2012) 16S rRNA terminal restriction fragment length polymorphism for the characterization of the nasopharyngeal microbiota. *PloS one*. **7**(12), e52241.

Buerger, S., Spoering, A., Gavrish, E., Leslin, C., Ling, L.L., Epstein, S.S. (2012) Microbial scout hypothesis, stochastic exit from dormancy, and the nature of slow growers. *Applied and environmental microbiology*. **78**(9), 3221–3228.

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M., Jolivet-Gougeon, A. (2012) Emergence of resistance to antibacterial agents: The role of quaternary ammonium compounds - A critical review. *International Journal of Antimicrobial Agents*. **39**(5), 381–389.

BusinessDesk (2015) Danone Nutricia posts full-year loss, blames Fonterra scare. *New Zealand Herald*, 1–2. [online]. Available from: http://www.nzherald.co.nz/business/news/article.cfm?c_id=3&objectid=11462367 [Accessed July 31, 2015].

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010) QIIME allows analysis of high- throughput community sequencing data. *Nature Publishing Group*. **7**(5), 335–336.

Carr, L.P., Ponoemon, L.A. (1994) The behavior of quality costs: classifying the confusion. *Journal of cost management practices*. **Summer**, 26–34.

Carroll, C. (2006) Defying a Reputational Crisis – Cadbury's Salmonella Scare: Why are Customers Willing to Forgive and Forget? *Corporate Reputation Review*. **12**(1), 64–82.

CellFacts2014_Ltd. (2015) CellFacts - Rapid Microbiology Designed for Manufacturing. [online]. Available from: http://www.cellfacts2014.com/ [Accessed June 20, 2015].

Chauvel, A.M., Andre, Y.A. (1985) Quality costs: better prevent than cure. In J. Campanella, ed. *Quality costs: Ideas and applications*. Milwaukee, WI: ASQC Quality Press, pp. 292–298.

Codex_Alimentarius (2013) Pesticide Residues in Food and Feed: 2-phenylphenol. *FAO/WHO*. [online]. Available from: http://www.codexalimentarius.net/pestres/data/pesticides/details.html?d-16497-o=2&d-16497-s=3&id=56&print=true [Accessed July 17, 2015].

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., Tiedje, J.M. (2005) The Ribosomal Database Project (RDP-II): Sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research*. **33**(DATABASE ISS.), 294–296.

Connon, S.A., Giovannoni, S.J. (2002) High-Throughput Methods for Culturing Microorganisms in Very-Low-Nutrient Media Yield Diverse New Marine Isolates. *Applied and environmental microbiology*. **68**(8), 3878–3885.

Courtois, S., Frostegård, Å., Göransson, P., Depret, G., Jeannin, P., Simonet, P. (2001) Quantification of bacterial subgroups in soil: Comparison of DNA extracted directly from soil or from cells previously released by density gradient centrifugation. *Environmental Microbiology*. **3**(7), 431–439.

Cowan, A.E., Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., Setlow, P. (2004) Lipids in the inner membrane of dormant spores of Bacillus species are largely immobile. *Proceedings of the National Academy of Sciences*. **101**, 7733–7738.

Cruaud, P., Vigneron, A., Lucchetti-Miganeh, C., Ciron, P.E., Godfroy, A., Cambon-Bonavita, M.A. (2014) Influence of DNA extraction method, 16S rRNA targeted hypervariable regions, and sample origin on microbial diversity detected by 454

pyrosequencing in marine chemosynthetic ecosystems. *Applied and Environmental Microbiology*. **80**(15), 4626–4639.

Culman, S.W., Gauch, H.G., Blackwood, C.B., Thies, J.E. (2008) Analysis of T-RFLP data using analysis of variance and ordination methods: a comparative study. *Journal of microbiological methods*. **75**(1), 55–63.

Davey, H.M. (2011) Life, death, and in-between: meanings and methods in microbiology. *Applied and environmental microbiology*. **77**(16), 5571–6.

Davey, M.E., Toole, G.A.O. (2000) Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiology and molecular biology reviews*. **64**(4), 847–867.

Davis, K.E.R., Joseph, S.J., Peter, H., Janssen, P.H. (2005) Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. *Applied and environmental microbiology*. **71**(2), 826–834.

De La Fuente, G., Belanche, A., Girwood, S.E., Pinloche, E., Wilkinson, T., Newbold, C.J. (2014) Pros and cons of ion-torrent next generation sequencing versus terminal restriction fragment length polymorphism T-RFLP for studying the rumen bacterial community. *PLoS ONE*. **9**(7).

Delmont, T.O., Robe, P., Cecillon, S., Clark, I.M., Constancias, F., Simonet, P., Hirsch, P.R., Vogel, T.M. (2011) Accessing the soil metagenome for studies of microbial diversity. *Applied and environmental microbiology*. **77**(4), 1315–24.

Delmont, T.O., Robe, P., Clark, I.M., Simonet, P., Vogel, T.M. (2011) Metagenomic comparison of direct and indirect soil DNA extraction approaches. *Journal of microbiological methods*. **86**(3), 397–400.

Denyer, S.P. (1995) Mechanisms of Action of Antibacterial Biocides. *International Biodeterioration & Biodegradation*, 227–245.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*. **72**(7), 5069–5072.

Desjardins, E., Beaulieu, C. (2003) Identification of bacteria contaminating pulp and a paper machine in a Canadian paper mill. *Journal of industrial microbiology & biotechnology*. **30**(3), 141–145.

Di Maiuta, N. (2010) *Characterisation of Mixed Microbial Populations in White Mineral Dispersions*. University of Warwick.

Di Maiuta, N., Hubschmid, S., Giuliani, N., Schwarzentruber, P., Dow, C.S. (2009) Microbial degradation of formaldehyde in white mineral dispersions preserved with formaldehyde-releasing biocides. *International Biodeterioration & Biodegradation*. **63**(6), 769–777.

Di Maiuta, N., Schwarzentruber, P. (2011) Molecular detection of bacteria in calcium carbonate powder used in cosmetic formulations. *International journal of cosmetic science*. **33**(5), 426–31.

Di Maiuta, N., Schwarzentruber, P. (2012) Process for bacterial stabilizing of aqueous ground natural calcium carbonate and/or precipitated calcium carbonate and/or dolomite and/or surface-reacted calcium carbonate-comprising mineral preparations. . (19), 1–12.

Di Maiuta, N., Schwarzentruber, P. (2013) Process for stabilizing bacterial content of aqueous ground natural calcium carbonate and/or precipitated calcium carbonate and/or dolomite and/or surface-reacted calcium carbonate- comprising mineral preparations. . **1**(19), 1–24.

Di Maiuta, N., Schwarzentruber, P., Dow, C.S. (2011) Enhancement of the antimicrobial performance of biocidal formulations used for the preservation of white mineral dispersions. *Applied microbiology and biotechnology*. **89**(2), 429–39.

Dollhopf, S.L., Hashsham, S. a., Tiedje, J.M. (2001) Interpreting 16S rDNA T-RFLP Data: Application of Self-Organizing Maps and Principal Component Analysis to Describe Community Dynamics and Convergence. *Microbial ecology*. **42**(4), 495–505.

Dunbar, J., Ticknor, L.O., Kuske, C.R. (2001) Phylogenetic Specificity and Reproducibility and New Method for Analysis of Terminal Restriction Fragment Profiles of 16S rRNA Genes from Bacterial Communities. *Applied and environmental microbiology*. **67**(1), 190–197.

Dworkin, J., Shah, I.M. (2010) Exit from dormancy in microbial organisms. *Nature reviews. Microbiology*. **8**(12), 890–896.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England)*. **26**(19), 2460–1.

Elliott, J. (2012) Supply situation report: PCC stands the test of time. *Industrial Minerals*. (January), 1–5.

Epstein, S.S. (2009a) General Model of Microbial Uncultivability. In S. S. Epstein, ed. *Uncultivated Microorganisms*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 131–159.

Epstein, S.S. (2009b) Microbial awakenings. *Nature*. **457**(7233), 1083.

Epstein, S.S. (2013) The phenomenon of microbial uncultivability. *Current opinion in microbiology*. **16**(5), 636–42.

Ercole, C., Del Gallo, M., Mosiello, L., Baccella, S., Lepidi, A. (2003) Escherichia coli detection in vegetable food by a potentiometric biosensor. *Sensors and Actuators, B: Chemical*. **91**(1-3), 163–168.

European_Coatings (2014) Global biocides market to grow by 2020. *European Coatings Journal*. [online]. Available from: www.european-coatings.com/Markets-companies/Raw-materials-market/Global-biocides-market-to-grow-by-2020 [Accessed August 4, 2015].

Fadrosh, D.W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R.M., Ravel, J. (2014) An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome*. **2**(1), 6.

Faille, C., Bénézech, T., Midelet-Bourdin, G., Lequette, Y., Clarisse, M., Ronse, G.,

Ronse, A., Slomianny, C. (2014) Sporulation of Bacillus spp. within biofilms: A potential source of contamination in food processing environments. *Food Microbiology*. **40**, 64–74.

Feytis, A. (2011) GCC chases Asian paper. *Industrial Minerals*. (January), 1–9.

Fittipaldi, M., Nocker, A., Codony, F. (2012) Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of microbiological methods*. **91**(2), 276–89.

Flemming, H.-C., Meier, M., Schild, T. (2013) Mini-review: microbial problems in paper production. *Biofouling*. **29**(6), 683–96.

Fujimura, S., Sato, T., Hayakawa, S., Kawamura, M., Furukawa, E., Watanabe, A. (2015) Antimicrobial efficacy of combined clarithromycin plus daptomycin against biofilms-formed methicillin-resistant Staphylococcus aureus on titanium medical devices. *Journal of Infection and Chemotherapy*.

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P. (2005) Survival strategies of infectious biofilms. *Trends in Microbiology*. **13**(1), 34–40.

Fykse, E.M., Skogan, G., Davies, W., Olsen, J.S., Blatny, J.M. (2007) Detection of Vibrio cholerae by real-time nucleic acid sequence-based amplification. *Applied and Environmental Microbiology*. **73**(5), 1457–1466.

Gabor, E.M., de Vries, E.J., Janssen, D.B. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS microbiology ecology*. **44**(2), 153–63.

Garsin, D.A. (2010) Ethanolamine Utilisation in Bacterial Pathogens: Roles and Regulation. *Nature Reviews Microbiology*. **8**(4), 290–295.

Gavini, F., Holmes, B., Izard, D., Bej, A., Bernigaud, A., Jakubczak, E. (1989) Numerical Taxonomy of Pseudomonas alcaligenes, P. pseudoalcaligenes, P. mendocina, P. stutzeri, and Related Bacteria. *International Journal of Systematic Bacteriology*. **39**(2), 135–144.

Gefen, O., Balaban, N.Q. (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS microbiology reviews*. **33**(4), 704–717.

Gensberger, E.T., Sessitsch, A., Kostic, T. (2013) Propidium monoazide-quantitative polymerase chain reaction for viable Escherichia coli and Pseudomonas aeruginosa detection from abundant background microflora. *Analytical Biochemistry*. **441**(1), 69–72.

Gilbert, P., Moore, L.E. (2005) Cationic antiseptics: diversity of action under a common epithet. *Journal of Applied Microbiology*. **99**, 703–715.

Gilbert, P., Rickard, A.H., Mcbain, A.J. (2004) Biofilms and antimicrobial resistance. In A. P. Fraise, P. a. Lambert, & J.-Y. Maillard, eds. *Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization*. Oxford, UK: Blackwell Publishing Ltd., pp. 128–138.

Gonzalez, J.M., Portillo, M.C., Belda-Ferre, P., Mira, A. (2012) Amplification by PCR artificially reduces the proportion of the rare biosphere in microbial communities.

PloS one. **7**(1), e29973.

Gorman, S.P., Scott, E.M., Russell, A.D. (1980) Antimicrobial activity, uses and mechanism of action of glutaraldehyde. *The Journal of applied bacteriology*. **48**(2), 161–190.

Gould, G.W. (1996) Methods for preservation and extension of shelf life. *International Journal of Food Microbiology*. **33**(1), 51–64.

Gracias, K.S., McKillip, J.L. (2007) Nucleic acid sequence-based amplification (NASBA) in molecular bacteriology: A procedural guide. *Journal of Rapid Methods and Automation in Microbiology*. **15**(3), 295–309.

Gray, J. (2013) Fonterra botulism crisis was false alarm. *New Zealand Herald*. [online]. Available from: http://www.nzherald.co.nz/business/news/article.cfm?c_id=3&objectid=11115784 [Accessed August 1, 2015].

Grieshaber, D., MacKenzie, R., Vörös, J., Reimhult, E. (2008) Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors*. **8**(3), 1400–1458.

Grocery_Manufacturers_Association (2011) *Capturing Recall Costs*.

Guérin-Méchin, L., Dubois-Brissonnet, F., Heyd, B., Leveau, J.Y. (2000) Quaternary ammonium compound stresses induce specific variations in fatty acid composition of Pseudomonas aeruginosa. *International Journal of Food Microbiology*. **55**(1-3), 157–159.

Guérin-Méchin, L., Dubois-Brissonnet, F., Heyd, B., Leveau, J.Y. (1999) Specific variations of fatty acid composition of Pseudomonas aeruginosa ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *Journal of Applied Microbiology*. **87**(5), 735–742.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D. V, Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S.K., Sodergren, E.J., Methé, B.A., DeSantis, T.Z., Petrosino, J.F., Knight, R., Birren, B.W. (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome research*. **21**(3), 494–504.

Hall, B.G. (2013) Building phylogenetic trees from molecular data with MEGA. *Molecular Biology and Evolution*. **30**(5), 1229–1235.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. (2001) Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*. **4**(1), 9–18.

Harrington, H.J. (1987) *Poor Quality Cost*. M. Dekker, ed. New York, NY: ASQC Quality Press.

Hattori, T., Mitsui, H., Haga, H., Wakao, N., Shikano, S., Gorlach, K., Kasahara, Y., El-Beltagy, A., Hattori, R. (1997) Advances in soil microbial ecology and the biodiversity. *Antonie van Leeuwenhoek*. **72**(1), 21–28.

Hawthorne, S.B., Kubátová, A., Gallagher, J.R., Sorensen, J.A., Miller, D.J. (2005) Persistence and Biodegradation of Monoethanolamine and 2-Propanolamine at an Abandoned Industrial Site. *Environmental Science & Technology*. **39**(10), 3639–3645.

Heath, R.J., Li, J., Roland, G.E., Rock, C.O. (2000) Inhibition of the Staphylococcus aureus NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *Journal of Biological Chemistry*. **275**, 4654–4659.

Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E., Rock, C.O. (1999) Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *Journal of Biological Chemistry*. **274**, 11110– 11114.

Heath, R.J., Yu, Y.T., Shapiro, M.A., Olson, E., Rock, C.O. (1998) Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis. *Journal of Biological Chemistry*. **273**, 30316–30320.

Hiom, S.J. (2004) Preservation of Medicines and Cosmetics. In A. P. Fraise, P. a. Lambert, & J.-Y. Maillard, eds. *Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization*. Oxford, UK: Blackwell Publishing Ltd., pp. 388–407.

Hiom, S.J., Denyer, S.P., Talbot, C., Maillard, J.-Y., Spark, P., Smith, J. (2013) A preliminary investigation into the ability of three rapid microbiological methods to detect microorganisms in hospital intravenous pharmaceuticals. *PDA J Pharm Sci and Tech*. **67**(4), 376–386.

Hobby, G.L., Meyer, K., Chaffee, E. (1942) Observations on the mechanism of action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine*. **50**, 281–285.

Horikoshi, K. (2011) General Physiology of Alkaliphiles. In K. Horikoshi, ed. *Extremophiles Handbook*. Tokyo: Springer Japan, pp. 100–118.

Hu, Q., Lyu, D., Shi, X., Jiang, Y., Lin, Y., Li, Y., Qiu, Y., He, L., Zhang, R., Li, Q. (2014) A Modified Molecular Beacons–Based Multiplex Real-Time PCR Assay for Simultaneous Detection of Eight Foodborne Pathogens in a Single Reaction and Its Application. *Foodborne Pathogens and Disease*. **11**(3), 207–214.

Hubschmid, S. (2012) Microorganisms in coating colours – Diversity, diminution strategies, impacts on paper quality. *Coating International*.

Huet, A. a, Raygada, J.L., Mendiratta, K., Seo, S.M., Kaatz, G.W. (2008) Multidrug efflux pump overexpression in Staphylococcus aureus after single and multiple in vitro exposures to biocides and dyes. *Microbiology*. **154**(10), 3144–3153.

Huse, S.M., Dethlefsen, L., Huber, J.A., Mark Welch, D., Welch, D.M., Relman, D. a, Sogin, M.L. (2008) Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS genetics*. **4**(11), e1000255.

Hussain, M.A., Dawson, C.O. (2013) Economic Impact of Food Safety Outbreaks on Food Businesses. *Foods*. **2**, 585–589.

Illumina (2013) 16S metagenomic sequencing library preparation: Preparing 16S RNA gene amplicons for the Illumina MiSeq System.

Industrial_minerals (2015) IM May Price Movements. *Industrial Minerals*. **April**, 1–11.

Jackson, C.R., Harper, J.P., Willoughby, D., Roden, E.E., Churchill, P.F. (1997) A simple, efficient method for the separation of humic substances and DNA from

environmental samples. *Applied and environmental microbiology*. **63**(12), 4993–4995.

Janda, J.M., Abbott, S.L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology*. **45**(9), 2761–2764.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., Uyttendaele, M. (2010) Alternative microbial methods: An overview and selection criteria. *Food Microbiology*. **27**(6), 710–730.

Jones, K., Holtzman, A., Aronson, T., Glover, N., Boian, M., Froman, S., Brunk, C.F. (1996) Molecular Technique for Rapid Identification of Mycobacteria. *Microbiology*. **34**(1), 98–102.

Jones, M. V, Herd, T.M., Christie, H.J. (1988) Resistance of Pseudomonas aeruginosa to amphoteric and quaternary ammonium biocides. *Microbios*. **58**(234), 49–61.

Josephson, K.L., Gerba, C.P., Pepper, I.L. (1993) Polymerase Chain-Reaction Detection of Nonviable Bacterial Pathogens. *Applied and Environmental Microbiology*. **59**(10), 3513–3515.

Joux, F., Lebaron, P. (2000) Use of fluorescent probes to assess physiological functions of bacteriaat single-cell level. *Microbes and Infection*. **2**(12), 1523–1535.

Kaeberlein, T., Lewis, K., Epstein, S.S. (2002) Isolating 'Uncultivable' Microorganisms in Pure Culture in a Simulated Natural Environment. *Science*. **296**(5570), 1127–1129.

Kaprelyants, A.S., Gottschal, J.C., Kell, D.B. (1993) Dormancy in non-sporulating bacteria. *FEMS microbiology reviews*. **104**(3-4), 271–285.

Karanam, V.R., Reddy, H.P., Subba Raju, B. V, Rao, J.C., Kavikishore, P.B., Vijayalakshmi, M. (2008) Detection of indicator pathogens from pharmaceutical finished products and raw materials using multiplex PCR and comparison with conventional microbiological methods. *Journal of industrial microbiology & biotechnology*. **35**(9), 1007–18.

Kaulfers, P.M., Marquardt, A. (1991) Demonstration of formaldehyde dehydrogenase activity in formaldehyde-resistant Enterobacteriaceae. *FEMS microbiology letters*. **63**(2-3), 335–338.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., Barer, M.R. (1998) Viability and activity in readily culturable bacteria: A review and discussion of the practical issues. *Antonie van Leeuwenhoek*. **73**(2), 169–187.

Kennedy, N.A., Walker, A.W., Berry, S.H., Duncan, S.H., Farquarson, F.M., Louis, P., Thomson, J.M., Satsangi, J., Flint, H.J., Parkhill, J., Lees, C.W., Hold, G.L. (2014) The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLoS ONE*. **9**(2), 1–9.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., Lewis, K. (2004) Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters*. **230**(1), 13–18.

Khaneja, R., Perez-Fons, L., Fakhry, S., Baccigalupi, L., Steiger, S., To, E., Sandmann, G., Dong, T.C., Ricca, E., Fraser, P.D., Cutting, S.M. (2010) Carotenoids found in Bacillus. *Journal of Applied Microbiology*. **108**(6), 1889–1902.

Kim, M., Morrison, M., Yu, Z. (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. *Journal of microbiological methods*. **84**(1), 81–7.

Kim, S., Nakhai, B. (2008) The dynamics of quality costs in continuous improvement. *International Journal of Quality & Reliability Management*. **25**(8), 842–859.

Knapp, J.S., Jenkey, N.D., Townsley, C.C. (1996) The anaerobic biodegradation of diethanolamine by a nitrate reducing bacterium. *Biodegradation*. **7**(3), 183–9.

Knüsli, D., Friedli, R., Busenhart, J. (2015) *Food safety in a globalised world*. Zurich.

Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S., Bauer, T.W. (2010) Distinction between intact and antibiotic-inactivated bacteria by real-time PCR after treatment with propidium monoazide. *Journal of Orthopaedic Research*. **28**(9), 1245–1251.

Koch, A.L. (2001) Oligotrophs versus copiotrophs. *BioEssays*. **23**(7), 657–661.

Kolari, M., Nuutinen, J., Rainey, F.A., Salkinoja-Salonen, M.S. (2003) Colored moderately thermophilic bacteria in paper-machine biofilms. *Journal of industrial microbiology & biotechnology*. **30**(4), 225–238.

Kolari, M., Nuutinen, J., Salkinoja-Salonen, M.S. (2001) Mechanisms of biofilm formation in paper machine by Bacillus species: the role of Deinococcus geothermalis. *Journal of industrial microbiology & biotechnology*. **27**(6), 343–351.

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., Sass, H. (2005) Microbial Diversity in Coastal Subsurface Sediments : a Cultivation Approach Using Various Electron Acceptors and Substrate Gradients. *Applied and Environmental Microbiology*. **71**(12), 7819–7830.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology*. **79**(17), 5112–20.

Krulwich, T.A., Sachs, G., Padan, E. (2011) Molecular aspects of bacterial pH sensing and homeostasis. *Nature reviews. Microbiology*. **9**(5), 330–343.

Kummerle, N., Feucht, H., Kaulfers, P.M. (1996) Plasmid-mediated formaldehyde resistance in Escherichia coli: characterization of resistance gene. *Antimicrob. Agents Chemother.*. **40**(10), 2276–2279.

Lambert, R.J.W. (2004) Evaluation of antimicrobial efficacy. In A. P. Fraise, P. a. Lambert, & J.-Y. Maillard, eds. *Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization*. Oxford, UK: Blackwell Publishing Ltd., pp. 345–360.

Lambert, R.J.W., Johnston, M.D., Hanlon, G.W., Denyer, S.P. (2003) Theory of antimicrobial combinations: Biocide mixtures - Synergy or addition? *Journal of*

Applied Microbiology. **94**(4), 747–759.

Law, J.W.-F., Ab Mutalib, N.-S., Chan, K.-G., Lee, L.-H. (2015) Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in Microbiology*. **5**(January), 1–19.

Lay, J.O. (2001) MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews*. **20**(4), 172–194.

Lazarova, V., Manem, J. (1995) Biofilm characterization and activity analysis in water and wastewater treatment. *Water research*. **29**(10), 2227–2245.

Lee, S., Kemp, P.F. (1994) Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. *Limnology and Oceanography*. **39**(4), 869–879.

Leem, H., Shukla, S., Song, X., Heu, S., Kim, M. (2014) An Efficient Liposome-Based Immunochromatographic Strip Assay for the Sensitive Detection of S almonella Typhimurium in Pure Culture. *Journal of Food Safety*. **34**(3), 239–248.

Leggett, M.J., Mcdonnell, G., Denyer, S.P., Setlow, P., Maillard, J.-Y. (2012) Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology*. **113**(3), 485–498.

Lennon, J.T., Jones, S.E. (2011) Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature reviews. Microbiology*. **9**(2), 119–130.

Levy, S.B. (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. *Journal of Applied Microbiology*. **92**(Symposium Supplement), 65S–71S.

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nature reviews. Microbiology*. **5**(1), 48–56.

Lindahl, V., Bakken, L.R. (1995) Evaluation of methods for extraction of bacteria from soil. *FEMS Microbiology Ecology*. **16**(2), 135–142.

Lindner, W. (2005) Surface coatings. In W. Paulus, ed. *Directory of Microbicides for the Protection of Materials*. Dordrecht: Springer Netherlands, pp. 347–375.

Liu, F., Li, Y., Su, X.-L., Slavik, M.F., Ying, Y., Wang, J. (2007) QCM immunosensor with nanoparticle amplification for detection of Escherichia coli O157:H7. *Sensing and Instrumentation for Food Quality and Safety*. **1**(4), 161–168.

Liu, W.-T., Marsh, T.L., Cheng, H., Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and environmental microbiology*. **63**(11), 4516–22.

Liu, Z., DeSantis, T.Z., Andersen, G.L., Knight, R. (2008) Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. *Nucleic Acids Research*. **36**(18), 1–11.

Liu, Z., Lozupone, C.A., Hamady, M., Bushman, F.D., Knight, R. (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Research*. **35**(18), 1–10.

Long, F., Zhu, A., Shi, H. (2013) Recent advances in optical biosensors for

environmental monitoring and early warning. *Sensors*. **13**(10), 13928–13948.

Lukow, T., Dunfield, P., Liesack, W. (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic. *FEMS Microbiology Ecology*. **32**(3), 241–247.

Mackey, B.M. (2000) Injured Bacteria. In B. M. Lund, T. C. Baird-Parker, & G. W. Gould, eds. *The microbiological safety and quality of food*. Gaithersburg, Md.: Aspen Publishers, pp. 315–341.

Madden, T. (2002) The BLAST Sequence Analysis Tool. In J. McEntyre & J. Ostell, eds. *The NCBI Handbook[internet]*. Bethesda (MD): National Center for Biotechnology Information (US), pp. 1–15.

Maillard, J.-Y. (2002) Bacterial target sites for biocide action. *Journal of Applied Microbiology*. **92**(Symposium Supplement), 16S–27S.

Manning, L. (2007) Food safety and brand equity. *British Food Journal*. **109**(7), 496–510.

Marsh, T.L. (1999) Terminal Restriction Fragment Length Polymorphism: an emerging method for characterizing diversity among homologous populations of amplicons. *Current Opinions in Microbiology*. **2**(3), 323–327.

Maseda, H., Hashida, Y., Konaka, R., Shirai, A., Kourai, H. (2009) Mutational upregulation of a resistance-nodulation-cell division-type multidrug efflux pump, SdeAB, upon exposure to a biocide, cetylpyridinium chloride, and antibiotic resistance in Serratia marcescens. *Antimicrobial agents and chemotherapy*. **53**(12), 5230–5235.

McAleece, N., Gage, J.D.G., Lambshead, P.J.., Paterson, G.L.J. (1997) BioDiversity Professional statistics analysis software. Jointly developed by the Scottish Association for Marine Science and the Natural History Museum London.

McDougald, D., Rice, S. a., Weichart, D., Kjelleberg, S. (1998) Nonculturability: Adaptation or debilitation? *FEMS Microbiology Ecology*. **25**(1), 1–9.

McMurry, L.M., McDermott, P.F., Levy, S.B. (1999) Genetic evidence that InhA of Mycobacterium smegmatis is a target for triclosan. *Antimicrobial Agents and Chemotherapy*. **43**, 711–713.

Michl, K., Gotsche, M., Rieger, J., Schmidt, K. (2007) Use of polyacrylic acids as grinding aids for calcium carbonate.

Microbiology_Consultants_LLC (2015a) Developing a Business Case for RMMs. [online]. Available from: http://rapidmicromethods.com/files/roi.php [Accessed July 28, 2015].

Microbiology_Consultants_LLC (2015b) RMMs and the Regulatory Environmental: Changing Acceptance Levels and Specifications. [online]. Available from: http://rapidmicromethods.com/files/regulatory_acceptance_levels.php [Accessed July 28, 2015].

Miller, M.J. (2010) The implementation of rapid microbiological methods - Part4. *European Pharmaceutical Review*. (4), 17–19.

Moir, A., Corfe, B.M., Behravan, J. (2002) Spore germination. *Cellular and Molecular Life Sciences*. **59**(3), 403–409.

Moldenhauer, J. (2008) Overview of Rapid Microbiological Methods. In M. Zourob, S. Elwary, & A. Turner, eds. *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*. New York: Springer Science+Business Media LLC, pp. 49–79.

More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C., Madsen, E.L. (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Applied and Environmental Microbiology*. **60**(5), 1572–1580.

Mori, Y., Notomi, T. (2009) Loop-mediated isothermal amplification (LAMP): A rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy*. **15**(2), 62–69.

Morimanno, L.R. (2013) Increasing calcium carbonate filler levels in toweling applciations. *Paper and Pulp International*. **55**(10), 41–44.

Mulet, M., Lalucat, J., García-Valdés, E. (2010) DNA sequence-based analysis of the Pseudomonas species. *Environmental Microbiology*. **12**(6), 1513–1530.

Munton, T.J., Russell, A.D. (1972) Effect of glutaraldehyde on the outer layers of Escherichia coli. *The Journal of applied bacteriology*. **35**(2), 193–199.

Murthy, P.S., Venkatesan, R. (2008) Industrial Biofilms and their Control. In H.-C. Flemming, P. S. Murthy, R. Venkatesan, & K. Cooksey, eds. *Marine and Industrial Biofouling*.

Nadal, A., Coll, A., Cook, N., Pla, M. (2007) A molecular beacon-based real time NASBA assay for detection of Listeria monocytogenes in food products: Role of target mRNA secondary structure on NASBA design. *Journal of Microbiological Methods*. **68**(3), 623–632.

Narrod, S.A., Jakoby, W.B. (1964) Metabolism of Ethanolamine. an Ethanolamine Oxidase. *The Journal of biological chemistry*. **239**(7), 2189–2193.

Navas-Molina, J. a, Peralta-Sánchez, J.M., González, A., McMurdie, P.J., Vázquez-Baeza, Y., Xu, Z., Ursell, L.K., Lauber, C.L., Zhou, H., Song, S.J., Huntley, J., Ackermann, G.L., Berg-Lyons, D., Holmes, S., Caporaso, J.G., Knight, R. (2013) *Advancing our understanding of the human microbiome using QIIME.* 1st ed. Elsevier Inc.

Nielsen, P., Fritze, D., Priest, F.G. (1995) Phenetic diversity of alkaliphilic Bacillus strains: Proposal for nine new species. *Microbiology*. **141**(7), 1745–1761.

Niessen, L., Luo, J., Denschlag, C., Vogel, R.F. (2013) The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. *Food Microbiology*. **36**(2), 191–206.

Nocker, A., Cheung, C.-Y., Camper, A.K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*. **67**(2), 310–320.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K. (2007) Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology*. **73**(16), 5111–5117.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*. **28**(12), E63.

Ogram, A., Sayler, G.S., Barkay, T. (1987) The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*. **7**, 57–66.

Oliver, J.D. (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*. **34**(4), 415–425.

Oliver, J.D. (2005) The Viable but Nonculturable State in Bacteria. *Journal of Microbiology*. **43**(S), 93–100.

Omachonu, V.K., Suthummanon, S., Einspruch, N.G. (2004) The relationship between quality and quality cost for a manufacturing company. *International Journal of Quality & Reliability Management*. **21**(3), 277–290.

Omidbakhsh, N., Ahmadpour, F., Kenny, N. (2014) How Reliable Are ATP Bioluminescence Meters in Assessing Decontamination of Environmental Surfaces in Healthcare Settings? *PLoS ONE*. **9**(6), e99951.

Omya_AG (2013) Omya AG webpage. [online]. Available from: http://www.omya.com/ [Accessed April 24, 2013].

Oppong, D., King, V.M., Zhou, X., Bowen, J. a (2000) Cultural and biochemical diversity of pink-pigmented bacteria isolated from paper mill slimes. *Journal of Industrial Microbiology and Biotechnology*. **25**(2), 74–80.

Ortega Morente, E., Fernández-Fuentes, M.A., Grande Burgos, M.J., Abriouel, H., Pérez Pulido, R., Gálvez, A. (2013) Biocide tolerance in bacteria. *International Journal of Food Microbiology*. **162**(1), 13–25.

Osborn, A.M., Moore, E.R.B., Timmis, K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental microbiology*. **2**(1), 39–50.

Pal, S., Ying, W., Alocilja, E.C., Downes, F.P. (2008) Sensitivity and specificity performance of a direct-charge transfer biosensor for detecting Bacillus cereus in selected food matrices. *Biosystems Engineering*. **99**(4), 461–468.

Pan, Y. (2011) Challenges and strategies for the application of rapid microbiological methods in the pharmaceutical industry - Supplement. *European Pharmaceutical Review*. **16**(5), 10–13.

Parikh, S.L., Xiao, G., Tonge, P.J. (2000) Inhibition of InhA, the enoyl reductase from Mycobacterium tuberculosis, by triclosan and isoniazid. *Biochemistry*. **39**, 7645–7650.

Parveen, S., Kaur, S., David, S. a W., Kenney, J.L., McCormick, W.M., Gupta, R.K. (2011) Evaluation of growth based rapid microbiological methods for sterility testing of vaccines and other biological products. *Vaccine*. **29**(45), 8012–23.

Paulus, W. (2005) Relationship between chemical structure and activity or mode of action. In W. Paulus, ed. *Directory of Microbicides for the Protection of Materials*. Dordrecht: Springer Netherlands, pp. 9–23.

Peris-Vicente, J., Carda-Broch, S., Esteve-Romero, J. (2015) Validation of Rapid Microbiological Methods. *Journal of Laboratory Automation*. **20**(3), 259–264.

Petrosino, J.F., Highlander, S.K., Luna, R.A., Richard, A., Versalovic, J. (2009) Metagenomic Pyrosequencing and Microbial Identification. *Clinical Chemistry*. **55**(5), 856–866.

Pinto, A.J., Raskin, L. (2012) PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. *PloS one*. **7**(8), e43093.

Poole, K. (2005) Efflux-mediated antimicrobial resistance. *Journal of antimicrobial chemotherapy*. **56**(1), 20–51.

Poole, K. (2004) Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection*. **10**(1), 12–26.

Poole, K. (2002) Mechanisms of bacterial biocide and antibiotic resistance. *Journal of Applied Microbiology*. **92**(Symposium Supplement), 55S–64S.

Purkiss, B.E. (1976) The microbiology of coolant/lubricants. *Industrial Lubrication and Tribology*. **28**(2), 44–49.

Rappé, M.S., Connon, S.A., Vergin, K.L., Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature*. **418**(6898), 630–633.

Rasooly, A., Herold, K.E. (2008) Food microbial pathogen detection and analysis using DNA microarray technologies. *Foodborne pathogens and disease*. **5**(4), 531–550.

Rättö, M., Suihko, M.-L., Siika-Aho, M. (2005) Polysaccharide-producing bacteria isolated from paper machine slime deposits. *Journal of Industrial Microbiology and Biotechnology*. **32**(3), 109–114.

Rittershaus, E.S.C., Baek, S.H., Sassetti, C.M. (2013) The normalcy of dormancy: Common themes in microbial quiescence. *Cell Host and Microbe*. **13**(6), 643–651.

Robe, P., Nalin, R., Capellano, C., Vogel, T.M., Simonet, P. (2003) Extraction of DNA from soil. *European Journal of Soil Biology*. **39**(4), 183–190.

Roberts, J. (2010) End user focus: Perfecting paper loading. *Industrial Minerals*. **May**, 2–3.

Rochex, A., Lebeault, J.-M. (2007) Effects of nutrients on biofilm formation and detachment of a Pseudomonas putida strain isolated from a paper machine. *Water research*. **41**(13), 2885–2892.

Rong-Hwa, S., Shiao-Shek, T., Der-Jiang, C., Yao-Wen, H. (2010) Gold nanoparticle-based lateral flow assay for detection of staphylococcal enterotoxin B. *Food Chemistry*. **118**(2), 462–466.

Roskill_Information_Services_Ltd (2012) *China drives Global Growth for Calcium Carbonate Demand*. London.

Russell, A.D. (2002) Antibiotic and biocide resistance in bacteria: Introduction. *Journal of Applied Microbiology*. **92**(Symposium Supplement), 1S–3S.

Russell, A.D. (1998) Assesssment of sporicidal efficacy. *International Biodeterioration and Biodegradation*. **41**(3-4), 281–287.

Russell, A.D. (2003) Challenge testing: Principles and practice. *International Journal of Cosmetic Science*. **25**(3), 147–153.

Russell, A.D. (2004) Factors influencing the efficacy of antimicrobial agents. In A. P. Fraise, P. A. Lambert, & J.-Y. Maillard, eds. *Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization*. Oxford, UK: Blackwell Publishing Ltd., pp. 98–122.

Sagar, K., Singh, S.P., Goutam, K.K., Konwar, B.K. (2014) Assessment of five soil DNA extraction methods and a rapid laboratory-developed method for quality soil DNA extraction for 16S rDNA-based amplification and library construction. *Journal of Microbiological Methods*. **97**(1), 68–73.

Saha, R., Donofrio, R.S. (2012) The microbiology of metalworking fluids. *Applied Microbiology and Biotechnology*. **94**(5), 1119–1130.

Saha, R., Spröer, C., Beck, B., Bagley, S. (2010) Pseudomonas oleovorans subsp. lubricantis subsp. nov., and reclassification of pseudomonas pseudoalcaligenes ATCC 17440T as later synonym of pseudomonas oleovorans ATCC 8062T. *Current Microbiology*. **60**(4), 294–300.

Salzburger, W. (1994) Microbial deterioration of materials - case histories and countermeasures for plastics and natural materials: Paper and additives. *Werkstoffe und Korrosion*. **45**, 180–184.

Sanchez, R. (2014) At sentencing, cantaloupe growers apologize for deadly listeria outbreak. *CNN*, 1–3. [online]. Available from: http://www.cnn.com/2014/01/28/justice/cantaloupe-listeria-deaths-sentencing/ [Accessed July 31, 2015].

Sandin, M., Allenmark, S., Edebo, L. (1992) Bacterial uptake of octyl ethanolamine increases with pH. *FEMS Microbiology Letters*. **91**(2), 147–152.

Sandin, M., Allenmark, S., Edebo, L. (1990) Selective toxicity of alkanolamines. *Antimicrobial Agents and Chemotherapy*. **34**(3), 491–493.

Sandrin, T.R., Goldstein, J.E., Schumaker, S. (2013) MALDI TOF MS profiling of bacteria at the strain level: A review. *Mass Spectrometry Reviews*. **32**(3), 188–217.

Schaefer, K., Jalbert, D., Kong, M. (2013) *World Pulp Monthly: Analysis and forecasts of the International Pulp market*.

Schmidt, T.M., Konopka, A.E. (2009) Physiological and Ecological Adaptations of Slow-Growing, Heterotrophic Microbes and Consequences for Cultivation. In S. S. Epstein, ed. *Uncultivated Microorganisms*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 101–120.

Scholtyssek, R. (2005) Protection of cosmetics and toiletries. In W. Paulus, ed. *Directory of Microbicides for the Protection of Materials*. Dordrecht: Springer Netherlands, pp. 263–286.

Schuelke&Mayr_GmbH (2015) Product insert-Mikrocount combi agar dip slides. , 1–8. [online]. Available from: http://www.schuelke.com/download/pdf/cint_lde-gb_mikrocount_combi_fol.pdf [Accessed July 27, 2015].

Schütte, U.M.E., Abdo, Z., Bent, S.J., Shyu, C., Williams, C.J., Pierson, J.D., Forney, L.J. (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Applied microbiology and biotechnology*. **80**(3), 365–380.

Schwarzentruber, P. (2003) *Microbiological Characterisation of White Pigment Slurries - A Strategy for Bacteria Management*. University of Warwick.

Schwarzentruber, P. (2007) *Protection of white mineral dispersions: Concepts, strategies, options and limitations*. Oftringen, Switzerland.

Schwarzentruber, P., Gane, P.A.C. (2005) Application of microbicides for the storage protection of mineral dispersions. In W. Paulus, ed. *Directory of Microbicides for the Protection of Materials. A handbook*. Dordrecht: Springer Netherlands, pp. 251–261.

Šedo, O., Vávrová, A., Vad'urová, M., Tvrzová, L., Zdráhal, Z. (2013) The influence of growth conditions on strain differentiation within the Lactobacillus acidophilus group using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling. *Rapid communications in mass spectrometry : RCM*. **27**(24), 2729–36.

Segers, P., Vancanneyt, M., Pot, B., Torck, U., Hoste, B., Dewettinck, D., Falsen, E., Kersters, K., De Vos, P. (1994) Classification of Pseudomonas diminuta Leifson and Hugh 1954 and Pseudomonas vesicularis Busing, Doll, and Freytag 1953 in Brevundimonas gen. nov. as Brevundimonas diminuta. *International Journal of Systematic Bacteriology*. **44**(3), 499–510.

Sergeev, N., Distler, M., Courtney, S., Al-Khaldi, S.F., Volokhov, D., Chizhikov, V., Rasooly, A. (2004) Multipathogen oligonucleotide microarray for environmental and biodefense applications. *Biosensors and Bioelectronics*. **20**(4), 684–698.

Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., Setlow, P. (2006) Role of dipicolinic acid in resistance and stability of spores of Bacillus subtilis with or without DNA-protective α/β-type small acid-soluble proteins. *Journal of Bacteriology*. **188**(11), 3740–3747.

Shama, G., Malik, D.J. (2013) The uses and abuses of rapid bioluminescence-based ATP assays. *International Journal of Hygiene and Environmental Health*. **216**(2), 115–125.

Shapiro, H.M. (2000) Membrane potential estimation by flow cytometry. *Methods*. **21**(3), 271–279.

Shen, Z., Hou, N., Jin, M., Qiu, Z., Wang, J., Zhang, B., Wang, X., Wang, J., Zhou, D., Li, J. (2014) A novel enzyme-linked immunosorbent assay for detection of Escherichia coli O157:H7 using immunomagnetic and beacon gold nanoparticles. *Gut Pathogens*. **6**(1), 14.

Sherburn, R.E., Large, P.J. (1999) Amine borate catabolism by bacteria isolated from contaminated metal-working fluids. *Journal of Applied Microbiology*. **87**(5),

668–675.

Shyu, C., Soule, T., Bent, S.J., Foster, J.A., Forney, L.J. (2007) MiCA: a web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. *Microbial ecology*. **53**(4), 562–570.

Simões, M., Pereira, M.O., Vieira, M.J. (2005) Validation of respirometry as a short-term method to assess the efficacy of biocides. *Biofouling*. **21**(1), 9–17.

Sims, P.J., Waggoner, A.S., Wang, C., Hoffman, J.F. (1974) Studies on the Mechanism by Which Cyanine Dyes Measure Membrane Potential in Red Blood Cells and Phosphatidylcholine Vesicles. *Biochemistry*. **13**(16), 3315–3330.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*. **407**(6805), 762–764.

Siqueira, J.F., Sakamoto, M., Rosado, A.S. (2010) Microbial community profiling using terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE). In G. J. Seymour, M. P. Cullinan, & N. C. K. Heng, eds. *Oral Biology, Methods in Molecular Biology*. Methods in Molecular Biology. Totowa, NJ: Humana Press, pp. 71–85.

Snyder, B. (2015) Peanut Exec could face life sentence due to Salmonella outbreak. *Fortune Magazine*, 1–2. [online]. Available from: http://fortune.com/2015/07/24/stewart-parnell-salmonella/ [Accessed July 31, 2015].

Sohn, J.H., Kwon, K.K., Kang, J.-H., Jung, H.-B., Kim, S.-J. (2004) Novosphingobium pentaromativorans sp. nov., a high-molecular-mass polycyclic aromatic hydrocarbon-degrading bacterium isolated from estuarine sediment. *International journal of systematic and evolutionary microbiology*. **54**(5), 1483–1487.

Song, W., Kim, M., Tripathi, B.M., Kim, H., Adams, J.M. (2015) Predictable communities of soil bacteria in relation to nutrient concentration and successional stage in a laboratory culture experiment. *Environmental Microbiology*, n/a–n/a.

Spanka, R., Fritze, D. (1993) Bacillus cohnii sp. nov., a New, Obligately Alkaliphilic, Oval-Spore-Forming Bacillus Species with Ornithine and Aspartic Acid Instead of Diaminopimelic Acid in the Cell Wall. *International Journal of Systematic Bacteriology*. **43**(1), 150–156.

Staley, J.T., Konopka, A.E. (1985) Microorganisms in Aquatic and Terrestrial Habitats. *Annual review of microbiology*. **39**, 321–346.

Steves, B. (2014) *2014 Emerging Trends and Key Issues Report: Product recall and contamination risk management*.

Stickler, D.J. (2004) Bacterial resistance 6.1 Intrinsic resistance of Gram-negative bacteria. In A. P. Fraise, P. a. Lambert, & J.-Y. Maillard, eds. *Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization*. Oxford, UK: Blackwell Publishing Ltd., pp. 154–169.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W. (2002) Biofilms as Complex Differentiated Communities. *Annual Review of Microbiology*. **56**(1), 187–209.

Suominen, I., Suihko, M.-L., Salkinoja-Salonen, M.S. (1997) Microscopic study of migration of microbes in food-packaging paper and board. *Journal of industrial microbiology & biotechnology*. **19**(2), 104–113.

Sutton, S. (2011) Accuracy of Plate Counts. *Journal of Validation Technology*. **17**(3), 42–47.

Sutton, S., Jimenez, L. (2012) A Review of Reported Recalls Involving Microbiological Control 2004-2011 with Emphasis on FDA Considerations of 'Objectionable Organisms'. *American Pharmaceutical Review*. **15**(1), 42–57.

Suzuki, M., Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology*. **62**(2), 625–630.

Suzuki, M., Rappé, M.S., Giovannoni, S.J. (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Applied and Environmental Microbiology*. **64**(11), 4522–4529.

Suzuki, S., Hiraishi, A. (2007) Novosphingobium naphthalenivorans sp. nov., a naphthalene-degrading bacterium isolated from polychlorinated-dioxin-contaminated environments. *Journal of General and Applied Microbiology*. **53**(4), 221–228.

Tabata, A., Nagamune, H., Maeda, T., Murakami, K., Miyake, Y., Kourai, H. (2003) Correlation between Resistance of Pseudomonas aeruginosa to Quaternary Ammonium Compounds and Expression of Outer Membrane Protein OprR. *Antimicrobial Agents and Chemotherapy*. **47**(7), 2093–2099.

Takami, H. (2011) Genomics and Evolution of Alkaliphilic Bacillus Species. In K. Horikoshi, ed. *Extremophiles Handbook*. Tokyo: Springer Japan, pp. 184–211.

Thorpe, T.C., Wilson, M.L., Turner, J.E., DiGuiseppi, J.L., Willert, M., Mirrett, S., Reller, L.B. (1990) BacT/Alert: An automated colorimetric microbial detection system. *Journal of Clinical Microbiology*. **28**(7), 1608–1612.

Trias, J., Benz, R. (1994) Permeability of the cell wall of Mycobacterium smegmatis. *Molecular microbiology*. **14**(2), 283–290.

Turner, N.A., Harris, J., Russell, A.D., Lloyd, D. (2000) Microbial differentiation and changes in susceptibility to antimicrobial agents. *Journal of applied microbiology*. **89**(5), 751–759.

U.S._Food_and_Drug_Administration (2012) Omya Voluntarily Recalls Ground Limestone Products Due To Possible Health Risk. , 1. [online]. Available from: http://www.fda.gov/Safety/Recalls/ucm327185.htm [Accessed May 21, 2015].

Urakami, T., Araki, H., Suzuki, K., Komagata, K. (1993) Further Studies of the Genus Methylobacterium and Description of Methylobacterium aminovorans sp. nov. *International Journal of Systematic Bacteriology*. **43**(3), 504–513.

US_Department_of_Justice (2014) Peanut Corporation of America Former Officials and Broker Convicted on Criminal Charges Related to Salmonella Tainted Peanut Products. *U.S. Department of Justice Press Release*. [online]. Available from: http://www.fda.gov/iceci/criminalinvestigations/ucm419424.htm [Accessed August 1, 2015].

US_EPA (2006) *Reregistration Eligibility Decision for 2-phenylphenol and Salts (Orthophenylphenol or OPP)*.

US_FDA (2015) Bacteriological Analytical Manual (BAM). [online]. Available from: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm [Accessed July 14, 2015].

US_FDA (2013) FDA Authorizes Sunland to Resume Preparing and Distributing Food at Peanut Butter Plant. *US_FDA*. [online]. Available from: http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm320413.htm [Accessed August 2, 2015].

US_FDA (1997) HACCP Principles & Application Guidelines. *US_FDA*. [online]. Available from: http://www.fda.gov/Food/GuidanceRegulation/HACCP/ucm2006801.htm [Accessed August 2, 2015].

US_FDA (2012) Information on the Recalled Jensen Farms Whole Cantaloupes. *US_FDA*. [online]. Available from: http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm272372.htm .

Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiology Reviews*. **56**, 395–411.

Vaisanen, O.M., Mentu, J., Salkinoja-Salonen, M.S. (1991) Bacteria in food packaging paper and board. *Journal of Applied Bacteriology*. **71**, 130–133.

Vaisanen, O.M., Nurmiaho-Lassila, E.L., Marmo, S.A., Salkinoja-Salonen, M.S. (1994) Structure and composition of biological slimes on paper and board machines. *Applied and Environmental Microbiology*. **60**(2), 641–653.

Vaisanen, O.M., Weber, A., Bennasar, A., Rainey, F.A., Busse, H.-J., Salkinoja-Salonen, M.S. (1998) Microbial communities of printing paper machines. *Journal of Applied Microbiology*. **84**, 1069–1084.

Van Aken, B., Peres, C.M., Doty, S.L., Yoon, J.M., Schnoor, J.L. (2004) Methylobacterium populi sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (Populus deltoides x nigra DN34). *International Journal of Systematic and Evolutionary Microbiology*. **54**(4), 1191–1196.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C. (2010) An overview of foodborne pathogen detection: In the perspective of biosensors. *Biotechnology Advances*. **28**(2), 232–254.

Větrovský, T., Baldrian, P. (2013) The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. *PLoS ONE*. **8**(2), e57923.

Vikram, A., Bomberger, J.M., Bibby, K.J. (2015) Efflux as a Glutaraldehyde Resistance Mechanism in Pseudomonas fluorescens and Pseudomonas aeruginosa Biofilms. *Antimicrobial Agents and Chemotherapy*. **59**(6), 3433–3440.

Vilo, C., Dong, Q. (2012) Evaluation of the RDP Classifier Accuracy Using 16S rRNA Gene Variable Regions. *Metagenomics*. **1**, 1–5.

Vos, M., Quince, C., Pijl, A.S., de Hollander, M., Kowalchuk, G.A. (2012) A comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. *PLoS ONE*. **7**(2), e30600 1–8.

Waghela, C.B. (2014) Paradigm shift in wet stage protection of water based paints. *Paint India*. (April), 74–86.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*. **73**(16), 5261–5267.

Wang, X.W., Zhang, L., Jin, L.Q., Jin, M., Shen, Z.Q., An, S., Chao, F.H., Li, J. (2007) Development and application of an oligonucleotide microarray for the detection of food-borne bacterial pathogens. *Applied Microbiology and Biotechnology*. **76**(1), 225–233.

Wang, Y., Ye, Z., Si, C., Ying, Y. (2011) Subtractive Inhibition Assay for the Detection of E. coli O157:H7 Using Surface Plasmon Resonance. *Sensors*. **11**(12), 2728–2739.

Wei, D., Oyarzabal, O.A., Huang, T.-S., Balasubramanian, S., Sista, S., Simonian, A.L. (2007) Development of a surface plasmon resonance biosensor for the identification of Campylobacter jejuni. *Journal of Microbiological Methods*. **69**(1), 78–85.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*. **173**(2), 697–703.

Welker, M. (2011) Proteomics for routine identification of microorganisms. *Proteomics*. **11**(15), 3143–3153.

Werner, J.J., Koren, O., Hugenholtz, P., DeSantis, T.Z., Walters, W.A., Caporaso, J.G., Angenent, L.T., Knight, R., Ley, R.E. (2012) Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *The ISME Journal*. **6**(1), 94–103.

Wilkes, J.G., Tucker, R.K., Montgomery, J.A., Cooper, W.M., Sutherland, J.B., Buzatu, D.A. (2012) Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for Escherichia coli serotype O157 by flow cytometry. *Food Microbiology*. **30**(1), 281–288.

Williams, G.R., Callely, A.G. (1982) The Biodegradation of Diethanolamine and Triethanolamine by a Yellow Gram-negative Rod. *Journal of General Microbiology*. **128**, 1203–1209.

Williams, T.M. (2007) The Mechanism of Action of Isothiazolone Biocides. *PPChem*. **9**(1), 14–22.

Williamson, K.E., Kan, J., Polson, S.W., Williamson, S.J. (2011) Optimizing the indirect extraction of prokaryotic DNA from soils. *Soil Biology and Biochemistry*. **43**(4), 736–748.

Wilson, I. (2008) Asian GCC loads up. *Industrial Minerals*. **September**, 1–10.

Wilson, I. (2015) GCC resources, production and markets of Asia. *Industrial*

Minerals. **April**, 1–10.

Winkowski, K. (2004) Biocide Optimization: Blends of Actives. *Paint & Coatings Industry*, 72–76.

Wu, M., Maier, E., Benz, R., Hancock, R.E.W. (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. *Biochemistry*. **38**(22), 7235–7242.

Xu, H.-S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., Colwell, R.R. (1982) Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. *Microbial Ecology*. **8**(4), 313–323.

Yoshimune, K., Morimoto, H., Hirano, Y., Sakamoto, J., Matsuyama, H., Yumoto, I. (2010) The obligate alkaliphile Bacillus clarkii K24-1U retains extruded protons at the beginning of respiration. *Journal of Bioenergetics and Biomembranes*. **42**(2), 111–116.

Yuan, S., Cohen, D.B., Ravel, J., Abdo, Z., Forney, L.J. (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PloS one*. **7**(3), e33865.

Yumoto, I., Hirota, K., Yoshimune, K. (2011) Environmental Distribution and Taxonomic Diversity of Alkaliphiles. In K. Horikoshi, ed. *Extremophiles Handbook*. Tokyo: Springer Japan, pp. 56–79.

Zhang, L., Dhillon, P., Yan, H., Farmer, S., Hancock, R.E.W. (2000) Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*. **44**(12), 3317–3321.

Zhao, F., Xu, K. (2012) Efficiency of DNA extraction methods on the evaluation of soil microeukaryotic diversity. *Acta Ecologica Sinica*. **32**(4), 209–214.

Zhao, X., Lin, C.-W., Wang, J., Oh, D.H. (2014) Advances in rapid detection methods for foodborne pathogens. *Journal of microbiology and biotechnology*. **24**(3), 297–312.

Zhou, B., Xiao, J., Liu, S., Yang, J., Wang, Y., Nie, F., Zhou, Q., Li, Y., Zhao, G. (2013) Simultaneous detection of six food-borne pathogens by multiplex PCR with a GeXP analyzer. *Food Control*. **32**(1), 198–204.

Appendices

Appendix I - Calculated cost per test for CFII

The following assumptions were made in calculating costs:

1. All prices are from 2011
2. Exchange rate GBP/EUR=1.13 (July 2011)
3. Hourly rate for the operator is £38/hr (€33/hr)
4. Shipping cost for consumables is ~£1300 per year (estimated from 2010 orders and deliveries)
5. Service contract is £5000 per year
6. There are 252 working days per year
7. Costs based on analyzing 15 samples/day
8. Certain consumables are used at the following frequency (when analyzing 15 samples/day):
    - 1 X 3L bag Cell-Lyte per week
    - 1 X 1L bottle of Cell-Pure per week
    - 1 X 1L Cell-Wash bottle every 2 months
    - 1 X 200mL Cell-Wash bag every 6 months
    - 1 X 2L Cell-Wash bag every 6 months

Table AI.1 - CFII cost per test assuming 15 samples analyzed during each run

	Costs (£)	
Consumable item	Histodenz	Sedimentation
2 mL tubes	0.03	-
1.5 mL tubes	0.08	0.08
7 mL bijoux	0.09	0.09
Histodenz	0.94	-
EDTA	0.02	-
SYTO 62	0.25	0.14
$diSC_3(5)$	0.11	0.06
Disruption Buffer	0.09	0.09
Acetic acid	-	0.02
Resuspension Buffer	0.02	-
Calibration beads	0.27	0.27
Cell-Lyte	0.02	0.02
Cell-Pure	0.13	0.13
Cell-Wash bag (small)	0.02	0.02
Cell-Wash bag (large)	0.04	0.04
Cell-Wash bottle	0.09	0.09
Cell-Lyte tubing	0.02	0.02
Shipping	0.28	0.28
Service contract	1.32	1.32
Total cost consumables per sample	3.82	2.68
Cost of operator time per sample	7.6	1.27
Total cost of test per sample (£)	**11.42**	**4.05**
(€)	**12.9**	**4.47**

Appendix II - Questionnaire for laboratory and quality managers

If possible please provide information/figures from 2008-2013.

General questions about products at your site

1. What volume of GCC slurry (in Mt) is sold?
2. What is the proportion of pH stabilized versus biocide-preserved GCC sold?
3. What is the annual usage (Mt) of biocide or pH stabilizer (e.g. AMP or MEA)?
4. How many customers do you supply to?

Frequency of customer complaints about contamination

5. What is the ratio of complaints to total GCC shipments or deliveries?
    - Is this ratio the same for pH stabilized and biocide-preserved slurries?

Managing complaints

6. Has your site ever had to change the terms of delivery to appease a customer's concerns about contamination? (For instance, dosing with biocide upon delivery into their storage tank). If so, what were the circumstances and what is/was the average added cost? (Cost/delivery or cost/volume and number of times this occurred in a given year. (Percentage of the total number of deliveries/year, or percentage of total Mt/year)).
    - What proportion of microbial complaints result in a re-negotiation of terms?
    - What proportion of customers has been through this process?

Handling rejected shipments

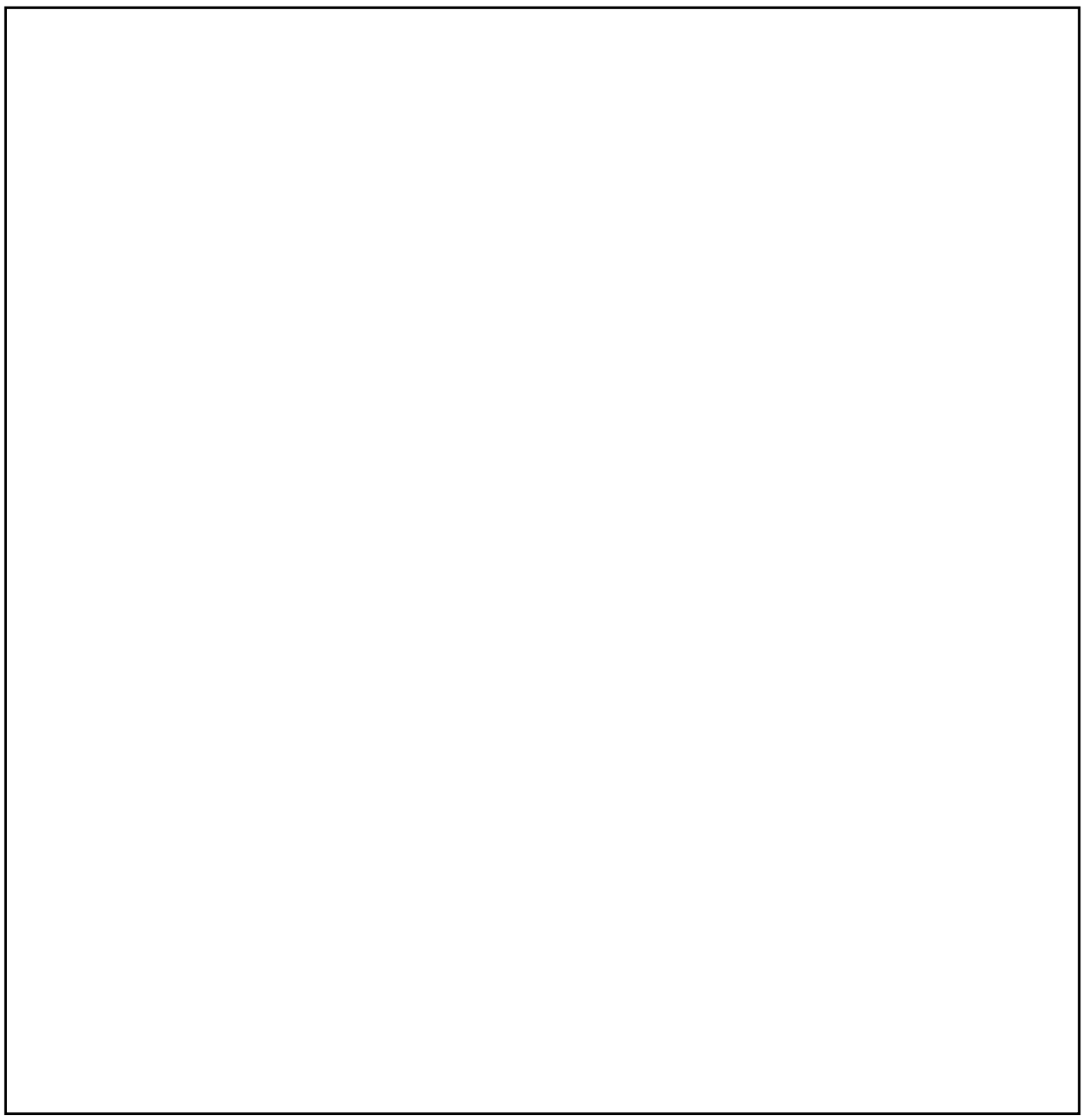
7. What percentage of rejected shipments is due to microbial contamination? (Percentage of total deliveries/year or percentage of Mt/year)
8. What proportion of returned slurry is successfully recovered by chemical treatments and resold?

(Optional) Opportunities for cost savings

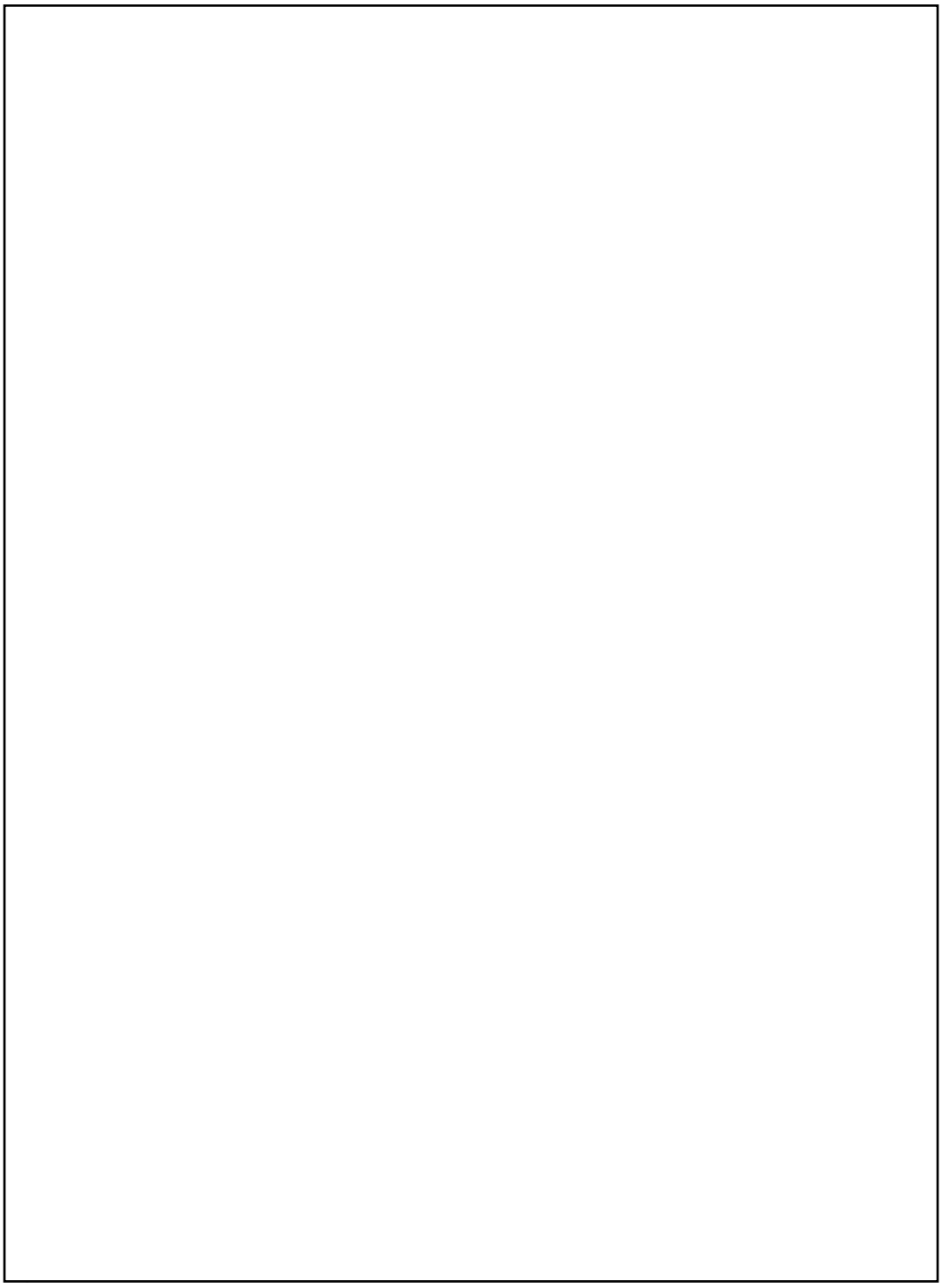
9. Where, if at all, do you feel there are opportunities to reduce costs associated with managing contamination? (For example, in reducing the frequency of post-treatments (NaOH, H_2O_2, biocide etc.), reducing costs associated with microbiology testing or plant hygiene, or reducing the frequency of customer complaints).
    - Could you provide a realistic estimate of potential cost savings per year and discuss how this might be achieved?

a. NOME - Responses to questionnaire

b. NLMJ - Responses to questionnaire



c. ATGU - Responses to questionnaire



d. ITAV - Responses to questionnaire

Appendix III - MiSeq N50X and N7XX primer sequences

Table AIII.1 - N50X and N7XX primers used in this study.
Modified from Illumina (2013)

Name	Sequence 5'→3'
N502-Rd1	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGT
N503-Rd1	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTCAGATGT
N504-Rd1	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTCAGATGT
N505-Rd1	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGATGT
N506-Rd1	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATGT
N507-Rd1	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGATGT
N508-Rd1	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTCAGATGT
N701-Rd2	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
N702-Rd2	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
N703-Rd2	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
N704-Rd2	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
N705-Rd2	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
N706-Rd2	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
N707-Rd2	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
N708-Rd2	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
N709-Rd2	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
N710-Rd2	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
N711-Rd2	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
N712-Rd2	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT

Appendix IV - QIIME analysis pipeline using MiSeq raw data (non-demultiplexed)

All steps are performed in the QIIME environment.

1. **Start with raw data** from Illumina MiSeq run, 4 fastq files: "…R1.fastq", "…R2.fastq", "…I1.fastq", "…I2.fastq"

2. **Combine Index 1 and Index 2 fastq files**

$ python *drag&drop combined_fastq_barcodes.py script file* Undetermined_S0…I1.fastq Undertermined_S0…I2.fastq combined_bc.fastq

↳ Output file: "combined_bc.fastq"

3. **Check mapping file**

$ validate_mapping_file.py –m *mapping_file.txt* –o validate_mapping_file_output

4. **Merge paired ends**

$ join_paired_ends.py –f Undertermined_S0…R1.fastq –r Undetermined_S0…R2.fastq –b combined_bc.fastq –o fastq-join_joined

↳ Output folder: "fastq-join_joined"

↳ 4 files: "fastqjoin.join_barcodes.fastq"; "fastqjoin.join.fastq"; "fastqjoin.un1.fastq"; "fastqjoin.un2.fastq"

5. **Demultiplexing and quality filtering**

$ split_libraries_fastq.py –i fastqjoin.join.fastq –b fastqjoin.join_barcodes.fastq –o slout_q30 –m *mapping_file.txt* --barcode_type 16 --store_qual_scores –q 29

↳ Output folder: "slout_q30" – *kept all reads with Q>30*

↳ 4 files:

- "histograms.txt" – *length of reads and numbers of reads at each length*
- "seqs.fna" – *sequences with sample names inserted into header*
- "seqs.qual" – *corresponding quality scores for .fna file*
- "split_library_log.txt" – *number of reads assigned to each sample*

6. **Concatenate seqs.fna files from multiple runs**

$ cat seqs.fna seqs.fna seqs.fna seqs.fna > all_seqs.fna

↳ Output file: "all_seqs.fna"

[optional] $ count_seqs.py –i all_seqs.fna – *to find out number of sequences that have been demultiplexed and passed quality filtering*

7. **OTU Picking**

$ pick_otus.py –i all_seqs.fna –o picked_otus_default

↳ Output folder: "picked_otus_default"

↳ 3 files:

- "all_clusters.uc"
- "all_otus.log" – *log file, number of OTUs*
- "all_otus.txt"

8. **Pick representative set**

$ pick_rep_set.py –i all_otus.txt –f all_seqs.fna –o rep_set1.fna

↳ Output file: "rep_set1.fna"

9. **Align sequences** [perform concurrently with Assign taxonomy]

$ align_seqs.py –i rep_set1.fna –t gg_13_8_otus/rep_set_aligned/97_otus.fasta –o $PWD/pynast_aligned_defaults/

↳ Output folder: “pynast_aligned_defaults”

↳ 3 files: “rep_set1_aligned.fasta”, “rep_set1_failures.fasta”, “rep_set1_log.txt”

10. **Assign taxonomy**

$ assign_taxonomy.py –i rep_set1.fna –r gg_13_8_otus/rep_set/97_otus.fasta –t gg_13_8_otus/taxonomy/97_otu_taxonomy.txt

↳ Output folder: “uclust_assigned_taxonomy”

↳ 2 files: “rep_set1_tax_assignments.log”, “rep_set1_tax_assignments.txt”

11. **Identify chimeric sequences**

$ identify_chimeric_seqs.py –m ChimeraSlayer –i rep_set1_aligned.fasta –a gg_13_8_otus/rep_set_aligned/97_otus.fasta –o chimeric_seqs.txt

↳ Output file: “chimeric_seqs.txt”

12. **Remove chimeric sequences from aligned sequences**

$ filter_fasta.py –f rep_set1_aligned.fasta –o non_chimeric_rep_set_aligned.fasta –s chimeric_seqs.txt –n

↳ Output file: “non_chimeric_rep_set_aligned.fasta”

13. **Filter alignment**

$ filter_alignment.py –i non_chimeric_rep_set_aligned.fasta –m lanemask_in_1s_and_0s –o filtered_alignment

↳ Output folder: “non_chimeric_rep_set_aligned_pfiltered.fasta”

14. **Make phylogenetic tree**

$ make_phylogeny.py –i non_chimeric_rep_set_aligned_pfiltered.fasta –o $PWD/rep_phylo.tre

↳ Output file: “rep_phylo.tre” view using “FigTree” program

15. **Make OTU table**

$ make_otu_table.py –i all.otus.txt –t rep_set1_tax_assignments.txt –o otu_table.biom

↳ Output file: “otu_table.biom”

16. **Filtering OTU table - 3 steps**

$ filter_otus_from_otu_table.py –i otu_table.biom –o otu_table_no_singletons.biom –n 2 *- discard all OTUs that are observed fewer than two times*

↳ Output file: “otu_table_no_singletons.biom”

$ filter_otus_from_otu_table.py –i otu_table_no_singletons.biom –o otu_table_ns_non_chimeric.biom –e chimeric_otus.txt *- remove all chimeric OTUs from the OTU table*

↳ Output file: “otu_table_ns_non_chimeric.biom”

$ filter_otus_from_otu_table.py –i otu_table_ns_non_chimeric.biom –o otu_table_ns_nc_minfiltered.biom --min_count_fraction 0.00005 *- discard OTUs with a number of sequences <0.005% of the total number of sequences to reduce spurious OTUs present in low abundance*

↳ Output file: “otu_table_ns_nc_minfiltered.biom”

17. **Stats of OTU table** - *view number of reads per sample assigned to the OTU table*

$ biom summarize-table –i otu_table.biom –o otu_table_summary.txt

- ↳ Output file: “otu_table_summary.txt”

18. **OTU heatmap**

$ make_otu_heatmap_html.py –i otu_table.biom –o OTU_heatmap

19. **Summarize OTU by category**

$ summarize_taxa_through_plots.py –i otu_table.biom –o wf_taxa_summary -m *mapping_file.txt*

- ↳ Output folder: “wf_taxa_summary”

20. **Convert biom file to txt** format to open in Excel

$ biom convert –i otu_table.biom –o otu_table_export.txt --b --header-key taxonomy

21. **Core diversity analyses**

$ core_diversity_analyses.py -i $PWD/otu_table.biom -o $PWD/core_output -m $PWD/mapping_file.txt -c Treatment;Season -t $PWD/phylo.tre -e 10000 (or desired sampling depth)

- ↳ Output folder: “core_output”
- ↳ Contains alpha and beta diversity analyses, group significance (kruskal-wallis)

Other helpful scripts:

$ compare_categories.py --method anosim -i weighted_unifrac_dm.txt -m mapping file -c category -o anosim_out

$ compute_core_microbiome.py -i biom file -o otu_table_core

$ split_otu_table.py -i otu_table.biom -m mapping.txt -f Category -o split_otu_table